Interactions between the endothelial mediator's nitric oxide and prostacyclin, and platelet $P2Y_{12}$ receptor blockade

A thesis submitted for the degree of Doctor of Philosophy in the Faculty of Medicine and Dentistry, Queen Mary University of London, 2017

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Declaration

I declare that the work reported in this thesis is all my own, with the following exceptions:

- cAMP and cGMP measurements reported in chapter 3 and chapter 4 were performed by Dr. Nicholas S. Kirkby, Department of Cardiothoracic Pharmacology, National Heart and Lung Institute, Imperial College London, UK.

- Platelet aggregation, platelet release and flow cytometry experiments reported in chapter 3, chapter 4 and chapter 5 were performed jointly with Dr. Paul C. Armstrong, Dr. Martina H. Lundberg, Mr Plinio M. Ferreira, Miss Melissa A. Hayman and Dr. Melissa V. Chan, Department of Translational Medicine and Therapeutics, William Harvey Research Institute, Queen Mary Univeristy of London.

- Rheometry experiments reported in chapter 7 were performed jointly with Dr. Matthew J. Lawrence, Dr. Lindsay A. D'Silva, Dr. Sophie N. Stanford and Dr. Ahmed Sabra, NISCHR Haemostasis Biomedical Research Unit, ABMU Health Board, Swansea, UK

- Scanning electron microscopy experiments reported in chapter 7 were performed by Dr. Lindsay A. D'Silva, NISCHR Haemostasis Biomedical Research Unit, ABMU Health Board, Swansea, UK

Publications

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Acknowledgements

I have been most fortunate to have met many friends and colleagues during the course of my PhD studies who helped me with my research and I will always be grateful to them. In particular I would like to recognise the considerable contribution of the following. Firstly, I express my immense gratitude to my supervisor Professor Tim Warner for his continuous support, motivation and guidance which helped me at every stage of my studies. An esteemed researcher who I will always hold in high regard who taught me much about research and life, asking insightful questions and offering invaluable advice. He was always there for me even through the hardest of times and never let me down. I thank Professor Arthur Tucker for his advice, reassurance and encouragement which gave me strength on many occasions and kept me going when things got tough. My sincere appreciation goes to Mrs Ivana Vojnovic whose immense kindness and thoughtfulness made such a difference to me so many times. Always ready and willing to do anything to help and support me and make me feel loved, her friendship I will always cherish as one of most special things to come from PhD years. I am very grateful to Dr Paul Armstrong for his patience, consideration and the assistance that he was always prepared to give me, I would not have been able to achieve what I have without him. My thanks also go to Dr Martina Lundberg and Dr Nicholas Kirkby for introducing a clinician into the laboratory and for their continued support thereafter. I am also grateful to the clinicians who welcomed me into their team and also to the volunteers and patients who participated in my research. A special thank you also goes to my close friends and colleagues Plinio, Will, Thomas and Michi who shared both the good and bad times with me. I feel very lucky to have made such strong friendships that I know will be life-long. My sincere gratitude goes to my viva examiners Professor Khalid Naseem and

Professor Peter Collins for the time and effort they invested in appraising my research. It was a privilege to discuss my thesis and learn from such highly regarded experts in the field. An inspirational experience that has motivated me to continue to pursue a career in academic Cardiology. Without the support of all of these individuals I would not have been able to complete this higher degree.

I am blessed with the support and love of family and friends who I have never needed more nor been more truly grateful to. Without your kindness, love and support I could not have continued. My deepest thanks and gratitude go to my father, Eric and mother, Armelle who gave me endless encouragement and were always eager to help me in any way that they could. And also to my grandparents, Yves and Marie-Louise whose love and support never faltered. My heartfelt appreciation and love go to Mike who shouldered these hardest of times with me to get me through, I simply wouldn't have survived without him and will be eternally grateful. Whilst it feels tragically bitter sweet and breaks my heart, I dedicate this PhD thesis to my beloved father and best friend and to my treasured grandfather and guardian angel both of whom I adored and both of whom I lost during the course of my studies. My world can never be complete or my smile truly happy again. Thank you for all that you did for me. I will forever love and miss you both so much.

Abstract

An often explored hypothesis is that in at risk patients the chances of experiencing a thrombotic event is associated with the level of $P2Y_{12}$ platelet blockade. However, studies have failed to show any benefits of *ex vivo* platelet function tests (PFT) and subsequent tailoring of treatment in patients receiving dual anti-platelet therapy (DAPT). This failure is possibly because these tests do not consider the environment in which platelets reside *in vivo*. Namely, that the strong synergies between $P2Y_{12}$ inhibitors, prostacyclin (PGI_2) and nitric oxide (NO) mean that *in vivo* platelet reactivity will be a function of the level of $P2Y_{12}$ receptor blockade and levels of endothelial-derived NO and PGI_2.

This thesis investigates the relationship between $P2Y_{12}$ blockade and endothelial mediators, emphasising the role of the endothelium in pathways of platelet activation. Initial *in vitro* platelet aggregation, release and flow cytometry experiments demonstrated the powerful, synergistic interactions between $P2Y_{12}$ blockade, NO and PGI_2. Immunoassays highlighted that cAMP rather than cGMP is the major driver of this synergy.

By giving healthy individuals standard $P2Y_{12}$ blockers it was determined that these *in vitro* observations hold true in man, with platelet responses powerfully influenced by the presence of NO and PGI_2. Furthermore, in patients with peripheral arterial disease (PAD) NO and PGI_2 strongly interacted with $P2Y_{12}$ blockade to inhibit platelet activation, adding further evidence that the *in vivo* environment is vital in determining platelet reactivity during DAPT.

Overall, this thesis highlights the importance of endothelial mediators in determining the therapeutic efficacy of $P2Y_{12}$ blockers, highlighting that endothelial function testing alongside *ex vivo* PFT could enhance risk prediction. It also provides a future basis to redefine optimal DAPT regimens in clinical populations, suggesting that alongside potent $P2Y_{12}$ receptor blockade, enhancement of inhibitory cyclic nucleotide pathways in platelets, rather than the co-administration of aspirin, could represent an effective therapeutic approach.

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Abbreviations

AA	Arachidonic acid
AC	Adenylyl cyclase
ACE	Angiotensin-converting enzyme
ACS	Acute coronary syndrome
ACh	Acetylcholine
ADP	Adenosine diphosphate
AF	Atrial fibrillation
ACCF	American College of Cardiology Foundation
AHA	American Heart Association
AMI	Acute myocardial infarction
ARU	Aspirin reactivity units
ASA	Aspirin
ATP	Adenosine triphosphate
CABG	Coronary artery bypass graft
CAD	Coronary artery disease
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanylyl monophosphate
COX	Cyclooxygenase
CPTP	Cyclopentyltriazolo-pyridine
CVA	Cerebrovascular accident
CYP P450	Cytochrome P450
DAG	Diacylglycerol
DAPT	Dual anti-platelet therapy
D_f	Fractal dimension
DM	Diabetes mellitus
DTI	Direct thrombin inhibitor
DVT	Deep vein thrombosis
ECG	Electrocardiogram
EDCF	Endothelium-derived vasoconstricting factor
EDHF	Endothelial-derived hyperpolarizing factor

EDRF	Endothelium-derived relaxing factor
eNOS	Endothelial nitric oxide synthase
EPCR	Endothelial protein C
ESC	European Society of Cardiology
ET	Endothelin
FA	Final aggregation
FcR	Fc receptor
FDA	Food and Drug Administration
FMD	Flow mediated dilatation
GC	Guanylyl cyclase
GI	Gastrointestinal
GP	Gel point
GP	Glycoprotein
GTN	Glyceryl trinitrate
GUSTO	Global Use of Strategies to Open Occluded Coronary Arteries
HDL	High-density lipoprotein
HTN	Hypertension
HTPR	High-on treatment platelet reactivity
HTRF	Homogenous time resolved fluorescence
ICAM	Intercellular adhesion molecule
ICH	Intracranial haemorrhage
IHD	Ischaemic heart disease
iNOS	Inducible nitric oxide synthase
INR	Internal normalised ratio
IPA	Inhibition of platelet aggregation
IP_3	Inositol-1,4,5-triphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
IV	Intravenous
LDL	Low-density lipoproteins
LDF	Laser Doppler flowmetry
LMWH	Low molecular weight heparin
LTA	Light transmission aggregometry

LTPR	Low on-treatment platelet reactivity
MACE	Major adverse cardiovascular event
MA	Maximal aggregation
MI	Myocardial infarction
MMP	Matrix metalloproteinase
NADPH	Nicotinamide adenine dinucleotide phosphate
NEJM	New England Journal of Medical
NF-κβ	Nuclear factor kappa-light-chain-enhancer
nNOS	Neuronal nitric oxide synthase
NOAC	Novel oral anti-coagulants
NSTEMI	Non-ST segment elevation myocardial infarction
NNH	Number needed to harm
NNT	Number needed to treat
OTPR	Optimal on-treatment platelet reactivity
PAD	Peripheral arterial disease
PAI	Plasminogen activator inhibitor
PAM	Prasugrel active metabolite
PAR	Protease-activated receptor
PAT	Pulse amplitude tonometry
PAU	Platelet aggregation units
PCI	Percutaneous coronary intervention
PDE	Phosphodiesterase
PORH	Post-occlusive reactive hyperaemia
PE	Pulmonary embolism
PFT	Platelet function test
PG	Prostaglandin
PGI_2	Prostaglandin I_2 (Prostacyclin)
PK	Protein kinase
PL	Phospholipase
POC	Point-of-care
PPACK	D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone
PPI	Proton pump inhibitor

PPP	Platelet poor plasma
PRI	Platelet reactivity index
PRP	Platelet rich plasma
PRU	$P2Y_{12}$ reactivity units
PSGL-1	P-selectin glycoprotein ligand 1
PS	Phosphodidylserine
PWA	Pulse wave analysis
RCT	Randomised controlled trials
RH	Reactive hyperaemia
RM	Relative mass
ROS	Reactive oxygen species
RRR	Relative risk reduction
SGP	Strain-gauge plethysmography
SNP	Sodium nitroprusside
ST	Stent thrombosis
STEMI	ST segment elevation myocardial infarction
TF	Tissue factor
TFPI	Tissue factor pathways inhibitor
T-PA	Tissue plasminogen activator
TIA	Transient ischaemic attack
TIMI	Thrombolysis in myocardial infarction'
TNF-α	Tumour necrosis factor alpha
TTP	Thrombotic thrombocytopenic
TVR	Target vessel revascularization
TX	Thromboxane
VASP	Vasodilator-stimulated phosphoprotein
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VTE	Venous thromboembolism
vWF	Von-Willebrand factor
VOP	Venous occlusion plethysmography
WHO	World Health Organisation

Chapter 1: Introduction

1.1 The cardiovascular system and haemostasis

The purpose of the cardiovascular system is to service the needs of the tissues. It serves to transport nutrients, including proteins, oxygen and hormones to and waste products from tissues, in order to maintain a homeostatic environment to allow optimal cell survival. This complex system consisting of the heart, fluid blood and the vasculature is regulated by complex mechanisms controlling cardiac output and arterial pressure to ensure blood flow corresponds to the tissues needs. It is divided into the systemic circulation which supplies all of the tissues of the body apart from the lungs and the pulmonary circulation and can be divided into functional parts. The left side of the heart acts as a pressure pump that pumps blood into the systemic arteries at a pressure sufficient enough to drive blood into the tissues. The right side of the heart pumps blood into the pulmonary arteries at a relatively lower pressure to supply the lungs. Arteries transport blood under high pressure to tissues with arterioles making up the last small branches of the arterial system acting as control valves that release blood into the capillaries to alter blood flow according to the needs of the tissue. Capillaries are thin walled and function to exchange fluid and substances between blood and the interstitial fluid. Blood is collected from the capillaries into venules which progressively increase in size to become veins that act not only as conduits for transport of blood to the heart but also as a controllable reservoir of blood depending on the organisms needs.

Of note, the greatest cross-sectional area of the cardiovascular system are the capillary beds and whilst the same volume of blood flows through each part of the circulation at any one time, the actual velocity of blood flow will be inversely proportional to the cross-sectional area of the vessel. For example, at rest blood will travel in the aorta at

approximately 33cm/sec however, in the capillary beds this velocity is reduced a 1000 fold to 0.3mm/sec. The cross-sectional areas and also the velocities of blood flow within the system are highlighted in table 1.1 and represent an important physical characteristic of the circulation.

Vessel	Cm^2
Aorta	2.5
Small arteries	20
Arterioles	40
Capillaries	2500
Venules	250
Small veins	80
Venae cavae	8

Table 1.1: Cross-sectional areas of the cardiovascular system [1]

Another fundamental principle of circulatory homeostasis is the existence of blood in a fluid state flowing in a pattern of laminar flow. I will now consider haemostasis, a major homeostatic mechanism of the cardiovascular system and a focus of this thesis.

The term haemostasis means the arrest of bleeding. The circulatory system has developed a range of haemostatic processes to maintain its integrity. Following vessel rupture, haemostasis is achieved by several mechanisms including vascular spasm, development of a platelet plug, blood clot formation as a result of blood coagulation and finally growth of fibrous tissue into the clot to allow wound healing to occur. A fundamental property of the cardiovascular system is how systemic imbalance will affect haemostasis differently between sites to allow for local clot formation yet keeping blood in other areas of the vasculature in a fluid state [2].

Three principal players in the processes of haemostasis in arterial vessels are the circulating blood platelets, the coagulation cascade and the endothelial cells that line the interior surface of blood vessels. Platelets respond to breakages in the arterial wall by sensing the underlying exposed proteins, adhering, activating and attracting in more platelets to rapidly build a platelet plug. This process also activates the clotting cascade to add insoluble fibrin strands to strengthen and bind together the growing thrombus. In parallel the cascade of factors leading to clot formation is limited by generation of anti-clotting processes that limit clot growth to ensure it remains focused on the local bleeding event.

Given the pivotal role of platelets in all aspects of haemostasis, in vascular spasm, coagulation and thrombosis it is not surprising that they are central to cardiovascular health and lie at the crux of many pathologies of the cardiovascular system. Platelets are therefore the targets of many therapeutic strategies in the fight against cardiovascular disease. Platelets will be the next focus of this thesis which will then go on to describe the process of haemostasis and underline the importance of the endothelium in cardiovascular homeostasis and in the control of platelet behaviour and how this is affected by anti-platelet therapies.

1.2 Platelets

Platelets are anucleate, discoid cells which were first described in the late 18th and early 19th centuries by scientists such as Osler, Bizzozero, Wright and Duke who recognised their anatomy and role in haemostasis and thrombosis. Since then our understanding of platelet biology and function has greatly expanded. We appreciate that the principal function of blood platelets is to ensure primary haemostasis and the cessation of bleeding following vascular injury but that unfortunately, these mechanisms overlap with the pathophysiological role of platelets in arterial thrombosis. It is also increasingly recognised that platelets are involved in other processes such as inflammation, angiogenesis and atherosclerosis.

1.2.1 Platelet formation and clearance

Platelets or thrombocytes are derived from megakaryocytes, highly specialised precursor cells descended from pluripotent stem cells that reside primarily within the bone marrow. Thousands of platelets are released from a single megakaryocyte during the process of thrombopoiesis, a process which is regulated by the cytokine thrombopoietin [3]. During maturation megakaryocytes undergo a cytoplasmic expansion phase and accumulate cytoplasmic proteins, organelles and granules that are essential for platelet function. The current model of platelet formation recognizes that mature megakaryocytes then extend long, branching processes, designated proplatelets via microtubule tracks into the vascular sinusoids of the bone marrow from where the platelets are then released into the bloodstream [4]. This process, in health is responsible for producing approximately 100 billion platelets a day to maintain a normal platelet count of 150-400×10^9/ml. A process which is intricately regulated to avoid spontaneous bleeding or arterial occlusion. The platelet lifetime, if it is not activated, is

approximately 10 days with older platelets changing in morphology by for example, losing volume and RNA and shedding receptors glycoprotein (GP) VI and GPIb. The exact mechanisms of platelet clearance are not fully understood but the majority are cleared by the reticuloendothelial system in the liver and spleen.

1.2.2 Platelet morphology

Mature platelets are discoid cell fragments measuring approximately 2 μm making up the smallest cellular blood component. They do not contain a nucleus so cannot transcribe DNA. However, they contain pre-mRNA and spliceosome machinery so are able to translate mRNA in to proteins. They comprise a phospholipid bilayer membrane on which the glycolyx rests and contain an extensive cytoskeleton, cannicular system, mitochondria and secretory granules comprising biologically active molecules [5]. Platelets contain three major types of secretory granules, alpha granules, dense granules and lysosomes. Platelet alpha granules are the most abundant platelet granule with 40-80 granules present in a single platelet containing many proteins including von-Willebrand factor (vWF), coagulation factor V, thrombospondin, P-selectin and fibrinogen [6]. Dense granules are less numerous and smaller than alpha granules and contain the adenine nucleotides adenosine triphosphate (ATP) and adenosine diphosphate (ADP), serotonin, pyrophosphate, calcium, and magnesium [7]. Lysosomes are the third type of platelet granule which contain acid hydrolases. Platelets contain few lysosomes and their function is not entirely understood.

Upon activation platelets undergo dramatic changes in morphology. In the shape change process following platelet activation, the cytoskeleton is rearranged with the projection of pseudopodia from the discoid membrane consisting of actin filaments and

tropomyosin [8]. Platelets lose their discoid shape, taking on an irregular spiky morphology with a much larger surface area driven by myosin phosphorylation [5]. Microtubules and granules are centralised during the shape change process in preparation for secretion.

1.3 Haemostasis

Haemostasis is the process which causes bleeding to stop and is the body's instinctive response to limit blood loss following injury. Haemostasis is maintained in the body by three intertwined mechanisms.

1.3.1 Vasoconstriction

This is the first response of blood vessels to local injury. Smooth muscle cells under control of the vascular endothelium contract. Vessel damage is sensed by local sympathetic nociceptors which promote vasoconstriction limiting blood loss. The increase in vascular resistance will increase the local shear forces on platelets and this, alongside their exposure to the subendothelial matrix will lead them to become activated. Activated platelets and the endothelium will release mediators such as serotonin, ADP and thromboxane (TX) A_2, all of which, increase the effect of vasoconstriction.

1.3.2 Primary haemostasis (Platelet aggregation)

Vitally, when the integrity of the vascular endothelium is breached platelets are exposed to pro-thrombotic proteins within the sub-endothelial matrix such as vWF or the primary platelet agonists collagen and thrombin. These triggers initiate a cascade of complex intracellular signalling pathways leading to the production of secondary platelet agonists, notably TXA_2 and ADP. These mediators constitute powerful positive feedback loops that greatly potentiate activation signals to drive formation of platelet-rich thrombi [9]. Primary haemostasis can be further divided into three stages, adhesion, activation and aggregation which are reviewed in detail below.

1.3.2.1 Adhesion

A key factor influencing platelet adhesion and aggregation is blood flow with distinct mechanisms operating in different shear conditions [10]. Notably, platelets are exposed to a broad range of haemodynamic conditions *in vivo* with low shear flow rates in venules and veins (<500 s^{-1}) to small arterioles (5000 s^{-1}) and shear rates over 40,000 s^{-1} in stenosed arteries [11].

Under normal conditions and laminar flow in the cardiovascular system, platelets due to their small size are marginalized to the periphery of the blood vessel by red blood cells. They travel or 'roll along' in close proximity to the endothelium allowing for close interactions between integrins and glycoproteins. This process is dependent upon the weak interaction between the A1 domain of immobilized vWF and the N-terminus of $GP1b_{\alpha}$, a portion of the GPIb-V-IX receptor on the platelet surface which promotes initial platelet tethering to the vessel wall. Though usually kept in a quiescent state within the circulation, platelets tethered at sites of vessel injury can become activated following exposure to sub-endothelial matrix components or other thrombogenic substrates. Notably, damage to the endothelium leads to the binding of vWF to exposed collagen which induces conformational changes allowing firm binding to GP1b facilitating stable adhesion and shape change.

1.3.2.2 Activation

The second stage of thrombus formation is described as platelet activation which can occur through a variety of platelet agonists and G-protein receptors described below. Platelets adhered to the site of vascular injury and exposed to the platelet agonists

reviewed below will become activated and through production of paracrine mediators activate and recruit freely circulating platelets in close proximity to the injury site.

1.3.2.2.1 Collagen

Sub-endothelial collagen fibrils that become exposed at areas of endothelial disruption play a major role in firm adhesion and promoting platelet arrest. There are two constitutively expressed receptors for collagen on platelets, GPVI and integrin $\alpha_2\beta_1$. GPVI is a low affinity collagen immunoglobulin receptor exclusively expressed on platelets and megakaryocytes. It exists in a complex with the Fc receptor (FcR) γ-chain which is essential for collagen induced platelet aggregation [12]. GPVI activates platelets through the phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) on the FcR γ-chain by the GPVI associated Src tyrosine kinases Lyn and Fyn [13]. This results in the activation of Syk which eventually causes phosphorylation and activation of phospholipase (PL) Cγ2 and the second messengers inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). This will lead to an increase in intracellular calcium and activate protein kinase (PK) C causing shape change, degranulation and the release and generation of the secondary mediators ADP and TXA_2, respectively [14]. The second collagen receptor integrin $\alpha_2\beta_1$ exists in a low affinity state requiring a conformational change to a high affinity state in order to bind collagen. Initial PKC activation by GPVI causes inside-out signalling and the switch of $\alpha_2\beta_1$ to a high affinity conformation binds collagen strongly and stabilizes platelet adhesion, reinforcing GPVI signalling [15].

1.3.2.2.2 Thrombin

Thrombin is the most potent platelet agonist, as well as being key in the coagulation cascade. Tissue factor (TF) and activation of factor XIIa are the initiators of thrombin

generation following injury vascular injury and trauma through activation of the coagulation cascade. Following factor Xa activation, thrombin is proteolytically cleaved from prothrombinin the blood stream. The interaction of factor Xa with factor Va greatly enhances the cleavage of thrombin from prothrombin and therefore, thrombin generation on the surface of platelets [16].

Activation of platelets by thrombin is mediated by the protease-activated receptors (PARs) PAR1 and PAR4 which are present on human platelets and have different affinities to thrombin. Activated PAR1 and PAR4 are both coupled to $G_{12/13}$ and G_q [17]. PAR1 is the major thrombin receptor with PAR4 existing as a lower-affinity thrombin receptor that can form a heterodimer with the high affinity PAR1 receptor [18]. PAR-1 has a high affinity for thrombin and leads to strong platelet activation at low concentrations of thrombin, whereas PAR-4 signals only at high concentrations of thrombin but for a prolonged duration [19]. The PAR receptors share a common proteolytic mechanism of activation with thrombin cleaving the N-terminal extracellular domain of the receptor at a specific site to create a new N-terminus that activates the receptor as a tethered ligand [20]. It is this tethered ligand mechanism that TRAP-amide, commonly used in the study of platelets, takes advantage of to induce platelet activation *in vitro* [21]. G_q activation then leads to the generation of the second messengers IP_3 and DAG leading to increases in extracellular calcium and PKC activation resulting in shape change and granule secretion and also in integrin αIIbβ3 activation [22]. $G_{12/13}$ activation also induces shape change through the Rho/Rho-kinase pathway by causing myosin light chain phosphorylation [16].

1.3.2.2.3 ADP

ADP is stored and released from platelet dense granules upon activation with other agonists such as thrombin, collagen or TXA_2 [23]. It acts as a strong autocrine and paracrine platelet activator and has an important role as a positive-feedback mediator for platelet activation. ADP acts upon two platelet receptors each coupled to a distinct G-protein with different effects. The $P2Y_1$ receptor which is linked to a G_q subunit and the $P2Y_{12}$ receptor which is linked to a G_i subunit. $P2Y_1$ mediates ADP-induced shape change and initiates weak, reversible aggregation through PLC which releases IP_3 from the membrane phospholipids and activates DAG, resulting in PKC activation and increased levels of intracellular calcium [24]. The $P2Y_{12}$ receptor amplifies and sustains platelet aggregation by inhibiting the activation of adenylyl cyclase (AC) which reduces intracellular cyclic adenosine monophosphate (cAMP) levels and by activating phosphoinositide 3-kinase [16]. Co-activation of both G protein-coupled receptors is essential for normal ADP-induced platelet aggregation [25].

1.3.2.2.4 TXA_2

TXA_2, like ADP is an important positive-feedback mediator during platelet activation and aggregation. TXA_2 is a prostanoid formed from arachidonic acid (AA) following its liberation from glycerophospholipids by PLA_2. Cyclooxygenase (COX) -1 and COX-2 catalyse the oxidation of AA to prostaglandin (PG) G_2 and then this is reduced by peroxidation to PGH_2. Thromboxane synthase then regulates the formation of TXA_2. TXA_2 acts on the G-protein coupled TP receptor, which exists in two forms: TP_α and TP_β [26]. Through this receptor linked to G_q, TXA_2 activates PLC which causes IP_3 and DAG signalling and mobilisation of intracellular calcium. This prostanoid is a mediator of platelet aggregation and thrombus formation, as well as a potent vasoconstrictor and it

also induces smooth muscle proliferation [27]. The actions of this pro-aggregatory prostanoid are considered to oppose those of the inhibitory endothelial prostanoid, prostacyclin (PGI_2), whose actions are reviewed in a further section of this thesis. The balance of these two mediators is considered a fundamental factor in the health of the cardiovascular system [28]. Chronic increase in TXA_2 is linked with the development of inflammatory diseases such as atherosclerosis and hypertension.

1.3.2.3 Aggregation

The ability of platelets to form a thrombus depends on their innate ability to aggregate. Platelet aggregation describes the process whereby activated platelets clump together forming a growing thrombus. This process occurs concomitantly to the platelet activation processes described above. Activation of the platelet receptor integrin αIIb/β3 is key to aggregation and particularly important in mediating the platelet-platelet interactions which must occur to facilitate thrombus formation. This integrin or GPIIb/IIIa as it also known, is the most abundant receptor on the surface of platelets with approximately 80,000-100,000 copies present per individual platelet [29]. This receptor is also present in alpha granules which become accessible following platelet stimulation [30].

Stimulation of this receptor can occur through a wide range of agonists acting through their separate receptors. Collagen, thrombin, ADP and TXA_2 detailed above are all examples. Central to GPIIb/IIIa's ability to mediate thrombus formation is its transition from a resting, low-affinity state to an active, high-affinity state revealing a RGD-binding sequence to bind its ligands. Activation then occurs by 'inside-out' signalling mediated by the GTPase Rap1b in response to both G_i and G_q signalling [31]. The active form of

αIIb/β3 can then bind divalent fibrinogen, multivalent vWF, prothrombin, fibronectin and other adhesion molecules which serve to bridge platelets together forming aggregates in the developing thrombus [32]. The key role of this receptor is highlighted as blockade of its functions inhibits the process of aggregation [33].

1.3.3 Secondary haemostasis (Coagulation)

Coagulation is the third and final step in haemostasis leading to the transformation of blood from a liquid to a gel like substance through activation of clotting factors and pro-coagulants. It describes the complex network of processes which result in the conversion of soluble fibrinogen into a fibrin mesh which reinforces the platelet plug.

The coagulation cascade of secondary haemostasis has two initial pathways which both lead to fibrin formation. These are the contact activation or intrinsic pathway and the TF or extrinsic pathway which converge leading to activation of factor X to factor Xa and activation of the final common pathway to produce fibrin [34]. The pathways are a series of reactions, in which inactive enzyme precursors and co-factors interact to become activated components that then catalyze the next reaction in the cascade. The intrinsic pathway is initiated with the activation of factor XII whereas the extrinsic pathway is started by the exposure and interaction of TF following vessel wall damage with factor VII [35]. Activation of the final common pathway will facilitate the formation of thrombin from prothrombin and the subsequent formation of fibrin from fibrinogen.

Platelets are central to several key coagulation processes. Activated platelets release a number of pro-coagulant molecules such as factor V and fibrinogen that are stored in alpha granules [36]. They also expose phosphatidylserine (PS) to support the function of

the prothrombinase complex greatly enhancing coagulation [37], interact with many coagulation factors [38] [39] and associate with TF [40]. Platelets are instrumental in providing a surface for large scale thrombin production [41] and are involved in fibrin formation and organisation with activated GPIIb/IIIa receptors regulating clot retraction [42].

Thus, from this we can recognise the importance of the physiological processes that take place in platelets to achieve haemostasis following compromise in the integrity of the vascular endothelium. We can also start to appreciate how these protective processes can turn pathological in atherothrombosis where atherosclerotic plaque rupture induces inappropriate rapid platelet activation leading to potential deadly thrombus formation. The next section of this thesis will go on to describe the physiology of the powerful endothelium and the fundamental role it plays in regulating cardiovascular homeostasis by maintaining platelets in a quiescent state within the vasculature. Following this I will then discuss how this critical balance can become disturbed in the process of atherothrombosis.

1.4 The Endothelium

The endothelium consists of a single layer of endothelial cells which line the lumen of every blood vessel providing a non-thrombogenic lining for the cardiovascular system. In adults, approximately ten trillion cells form an almost 1kg organ [43]. It is strategically located to separate the thrombogenic vascular wall from the circulation and from blood cells. Although once considered inert it is now recognised as being highly metabolically active. Endothelial cells are crucial in maintaining cardiovascular health and are involved in many physiological processes. Their vital roles in barrier function, vasomotor tone and haemostatic control in health and disease are further detailed below. The fundemental role of the endothelium in the regulation of platelet aggregation will be visited in more detail in the following section of this thesis.

In 1980 the seminal report from Furchgott and Zawadski described the obligatory nature of the endothelium in producing blood vessel relaxation and regulating vasomotor tone [44], work that earnt Furchgott the Nobel Prize in Medicine or Physiology in 1998. Since then, there has been a vast quantity of literature published in the area of blood vessel regulation by endothelial cells, building also on studies from the 1960s and 1970s by researchers such as Florey, Jaffe, Gimbrone and Vane [45]. Together these studies have characterised endothelial cells as a remarkably diverse cell population in both structure and function with differing properties in different vascular beds, in arterial, venous and microcirculations, and even within the same blood vessel with each cell displaying an inherent quality of being able to adapt to the needs of the underlying tissue [46, 47]. For example, endothelial cells lining post-capillary venules are primarily responsible for mediating leucocyte trafficking, whereas those found in the arterial system primarily

regulate vascular tone. Endothelial cells exert autocrine, paracrine and endocrine actions influencing smooth muscle cells, platelets and leucocytes [43]. A fundamental property of the endothelium is its phenotypic heterogeneity which is explained by its broad distribution receiving input signals from the extracellular environment varying throughout the vascular tree from astrocytes in the blood brain barrier to hepatocytes in the hepatic sinusoids [48]. In fact, different endothelial cells can generate different responses to the same stimulus. Endothelial cell phenotype also varies widely in health and disease and these cells are involved in many disease processes including atherosclerosis, hypertension and sepsis. The metabolic and synthetic function of endothelial cells is highlighted in figure 1.1.

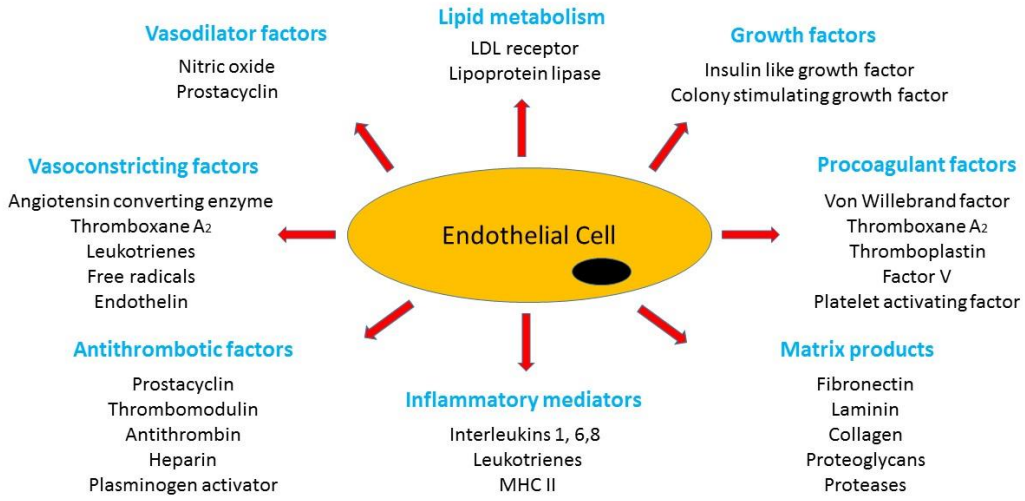


Figure 1.1: The metabolic and synthetic function of endothelial cells

The endothelium in most vascular beds is continuous and non-fenestrated (arteries and veins) but it can be continuous fenestrated and discontinuous/sinusoidal (capillaries) reflecting different functions in the body. It is mesodermal in origin and characterised by tight junctions at the lateral borders of each cell that restrict the passage of macromolecules. Its simple squamous cells are long flat cells, on average 20-40 µm long,

10-15 µm wide and only 0.1-0.5 µm thick [49]. It has a slow turnover rate of approximately 3 years but damage occurring from atherosclerosis for example, can lead to replacement by cells that do not possess the same properties as normal endothelial cells [50]. It acts as a selectively permeable barrier between intravascular and extravascular compartments and controls several physiological processes; allowing for interactions between circulating blood cells and the vessel wall, it regulates leukocyte trafficking by upregulation of adhesion molecules and chemokines (P-Selectin/E-Selectin/ vascular cell adhesion molecule 1 (VCAM-1)/ intercellular adhesion molecule 1 (ICAM-1)). It is also involved in cell survival and proliferation, vascular remodelling, metabolism, innate and acquired immunity, inflammation and angiogenesis. "You are only as old as your endothelium", the wise words of Dr. Rudolph Altschul in 1954 reflect the important contributions of the endothelium to cardiovascular physiology and pathology.

1.4.1 Barrier function

Macro molecules can cross the endothelial barrier by passing through endothelial cells themselves, through endothelial gaps, through intracellular junctions or via vesicular and caveolae transport. It is therefore, not surprising that caveolae are much more numerous in capillaries than they are in veins or arteries. Basal and inducible permeability are differently regulated across the vascular tree [51]. Increases in endothelial permeability to water and solutes are mediated by reorganisation of inter-cellular junctions by actin and myosin, which can be induced by inflammatory mediators such as thrombin and histamine, vascular endothelial growth factors (VEGF's) and 'low' shear stress [52]. A wide variety of injurious stimuli such as cigarettes or high fat diets contribute to endothelial dysfunction by increasing endothelial permeability leading to

lipid accumulation and the development of atherosclerotic plaques. Opposing this, protective stimuli for example, exercise by increasing blood flow and sheer stress leading to the release of vasoprotective molecules such as PGI_2 and nitric oxide (NO) preserve the endothelial barrier [49]. The endothelial glycocalyx, consisting of negatively charged proteins and proteoglycans also contributes to the protection of the vessel wall. Its destruction is linked to the loss of vascular responses, increased endothelial permeability and platelet aggregation and leads to the initiation and progression of atherosclerosis [53].

1.4.2 Vasomotor tone

The endothelium produces a number of vasodilator and vasoconstrictor substances which play a major role in the regulation of vasomotor tone. NO, PGI_2, endothelin (ET) and endothelial-derived hyperpolarizing factor (EDHF) are all powerful vasoactive substances released by endothelial cells in response to mechanical and humoral stimuli. The net balance of local endogenous healthy endothelium-derived relaxing factors (EDRFs) and endothelium-derived vasoconstrictor factors (EDCFs) maintains normal vascular tone. Examples of EDRFs are bradykinin, EDHF, NO and PGI_2. NO is a potent vasodilator important in maintaining the vasculature in a state of vasodilatation. Both endothelial constitutive and cytokine inducible nitric oxide synthase (eNOS and iNOS) which catalyse the production of NO are found in endothelial cells [54]. In smooth muscle cells, NO acting through secondary messengers inhibits calcium entry into the cells, as well as decreases myosin light chain phosphorylation to cause vasodilatation [55]. PGI_2 is also synthesized by endothelial cells in response to similar stimuli as for NO, as well as other stimuli such as platelet derived and epidermal growth factors and inflammatory mediators such as IL-1. It is also a powerful vasodilator. These two

powerful inhibitors will be revisited in more detail in the following section. ET, angiotensin and prostanoids such as TXA_2 are examples of EDCFs. ET is a vasoconstrictor produced by endothelial cells that has a marked effect on vasculature tone. It also stimulates cell proliferation. There are three types of ET but it is ET-1 which is synthesised by endothelial cells. There is a wide distribution of ET receptors in the body and in the vasculature. Stimulation of ET_A receptors in the vasculature causes vasoconstriction and stimulation of ET_B causes both vasoconstriction and vasodilatation, depending on the vascular bed [56]. There is considerable cross-talk between NO, PGI_2 and ET-1 in the control of vascular tone [57]. In pathological conditions such as hypertension (HTN) and diabetes mellitus (DM), an increase in EDCFs, as well as a decrease in EDRFs will favour vascular contraction and potentially, pathophysiological changes. In these conditions ET-1, as well as acting as a vasoconstrictor is implicated in proliferative responses and is also a chemoattractant for monocytes. Figure 1.2 represents the net balance of EDRFs and EDCFs in healthy and dysfunctional endothelium.

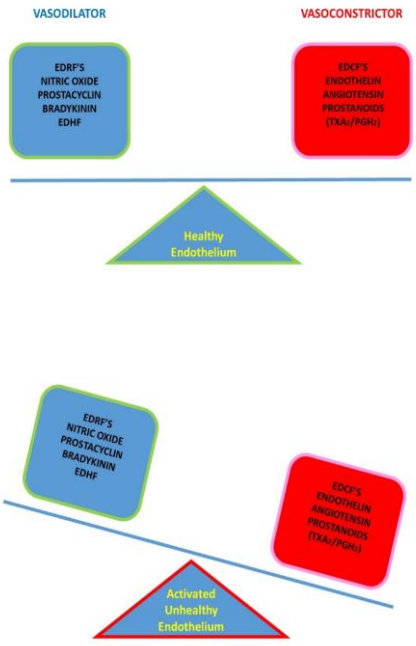


Figure 1.2: Healthy versus unhealthy endothelium - the net balance of EDRFs and EDCFs

1.4.3 Haemostatic balance

The endothelium is integrally involved in mediating haemostasis. Whilst the liver synthesizes a constant amount of pro-coagulant (fibrinogen, clotting cascade proteases) and anti-coagulant (protein C/antithrombin III) molecules, the endothelium produces a 'regulatable' supply. Endothelial cells produce a repertoire of endothelial-derived anticoagulant and procoagulant molecules which they display within the vasculature. Thrombomodulin, endothelial protein C (EPCR), heparin, CD39, eNOS and tissue factor pathways inhibitor (TFPI) are examples of the anticoagulants expressed by endothelial cells. Thrombomodulin is the major physiological buffer for the pro-coagulant effects of thrombin as it binds to the same site as fibrinogen, platelets and factor V so blocks all of these functions and instead activates anticoagulant protein C [43]. TF, factor VIII, plasminogen activator inhibitor (PAI) and vWF are procoagulant molecules produced by endothelial cells. The majority of vWF is synthesised in endothelial cells in one of two forms: vWF dimers, which are secreted into the plasma and sub-endothelial matrix and vWF multimers, which are stored in Weibel-Palade bodies in endothelial cells. The heterogeneity of the vascular tree is key, with each mediator differentially expressed both in a temporal and spatial manner explaining how systemic changes in the procoagulant and anticoagulant molecules listed above can lead to site specific thrombosis [2]. Specifically, for example, TFPI is mostly expressed in the capillaries, eNOS in the arterial circulation, vWF in the venous side of the circulation, thrombomodulin is widely expressed in all vessels apart from the cerebral circulation, tissue plasminogen activator (t-PA) is expression is more restricted to the pulmonary and cerebral arteries and EPCR is found in large veins and arteries. Therefore, endothelial cells at different sites within the vasculature will employ site specific

'formulas' of haemostatic proteins to maintain blood fluidity and promote limited blood clot formation following a breech in the integrity of the endothelium [48].

1.4.4 Endothelial cells in disease

In response to systemic inflammation the expression of these various factors changes in ways that differ between vascular beds [46]. Quiescent endothelial cells express an anticoagulant, anti-adhesive and vasodilatory phenotype, whereas activated or dysfunctional cells associated with disease states express pro-coagulant, pro-adhesive and vasoconstricting properties [58].

In the 1980s Gimbrone demonstrated that specific stimuli could induce expression of endothelial markers [59]. Since then it has become understood that endothelial cells can become activated to express inflammatory mediators such as IL-1 and tumour necrosis factor alpha (TNF-α) which correlate with this procoagulant activity. Therefore, endothelial cells are able to sense and respond to their extracellular environment. They can become adaptive whereby activation is a phenotypic response to an inflammatory stimulus, whereas endothelial dysfunction is thought of as a maladaptive response from otherwise adaptive responses that become excessive, sustained or misplaced representing a net liability to the host [58]. These cytokines, growth factors, lipids and enzymes modulate cell function and lead to lipid accumulation, vasoconstriction and promotion of thrombosis [43].

Haemodynamic forces are fundamental to endothelial cell biology,function and interactions with smooth muscle cells. It is well recognised that disturbed flow is atherogenic whereas steady laminar flow is atheroprotective. This is because flow

stimulates vasoactive genes and products, cytokines, adhesion molecules and growth factors from endothelial cells. Pathological flow will activate endothelial cells with a shift in the haemostatic balance to increased permeability and procoagulation increasing reactive oxygen species (ROS) and vasoconstrictors, whereas physiological flow will increase anticoagulants, vasodilators and antithrombotic molecules and decrease permeability [60]. It is suggested that endothelial cells in areas of disturbed flow are primed for activation with increased levels of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κβ) in their cytoplasm, with systemic imbalances leading to the translocation of NF-κβ into the nucleus resulting in increased expression of adhesion molecules and procoagulants [47]. Production of thrombomodulin and the vasodilators PGI_2 and ET-1, as well as NO through increased eNOS activity are all altered by shear stress [61-63]. Oxidative stress and ROS affect endothelial health and contribute to endothelial dysfunction and atherosclerosis. Oxidative stress results as a consequence of imbalances of anti-oxidative and pro-oxidative enzymes, for example heme oxygenase and nicotinamide adenine dinucleotide phosphate (NADPH), respectively leading to ROS production. ROS include the oxygen radicals (superoxide and hydroxyl radical) and non-radical oxygen derivatives (ozone and hydrogen peroxidase). Flow again contributes significantly to this balance with physiological flow inducing conditions with lower ROS and higher NO bioavailability and pathological flow tipping the balance towards higher ROS levels and reduced NO bioavailablity.

From this we can start to build a picture of the fundamental role of the endothelium in cardiovascular health and how this is altered in disease. In the following section I will consider specifically the role of the endothelium in regulating platelet function and how this becomes dysregulated in endothelial dysfunction and atherothrombosis.

1.5 The regulation of platelet function

Within the circulation platelets are subject to precise regulation to maintain them in a quiescent state to prevent their rapid and inappropriate activation [64]. The endothelium limits platelet activation by separating blood from the pro-thrombotic sub-endothelial matrix, as reviewed in a previous section. Crucially, as mentioned above endothelial cells are also responsible for the production of the two soluble inhibitory mediators that constantly bathe circulating platelets and limit their reactivity [45], the free radical NO and the inhibitory prostaglandin, PGI_2. Indeed, it is important to recognise that within the circulation each platelet is balanced by and under the influence of approximately 50 endothelial cells (e.g. 1.25 trillion platelets *vs.* 60 trillion endothelial cells in a 70 kg man).

1.5.1 PGI_2

PGI_2 is a prostanoid formed via the cyclooxygenase pathway, as described previously. It is synthesised by the enzyme PGI_2 synthase which is concentrated in the endothelium [65] and its production and release is highly dependent on the pulsatile and steady flow shear stress of blood within the vasculature [66] The actions of PGI_2 on platelets are mediated through the G-protein coupled prostacyclin (IP) receptor, which couples to G_s and when activated leads to activation of AC activity to produce an increase in intracellular cAMP [67]. PGI_2 acts upon neighbouring smooth muscle cells and platelets in a paracrine fashion causing its primary effects of vasodilatation and the inhibition of both platelets and smooth muscle cell proliferation, as previously highlighted [68]. Considered the physiological antagonist of TXA_2, PGI_2 produces protective anti-inflammatory, anti-thrombotic and anti-angiogenic effects within the cardiovascular system [69] and plays an important role in the prevention of cardiovascular diseases

such as atherosclerosis [68]. It has a short half-life of less than 3 minutes within the circulation but forms the stable metabolite 6-keto-$PGF_{1\alpha}$, which can be measured as a surrogate of PGI_2 activity. Importantly, this means that in *in vitro* tests of platelet function PGI_2 is no longer present or active.

1.5.2 NO

NO is produced from L-arginine by a family of enzymes named NOS of which there are three members; eNOS, iNOS and neuronal NOS (nNOS). As previously highlighted, NO is a potent inhibitor of platelet aggregation and vasoconstriction, released from endothelial cells following their activation by shear forces or via receptor mediated process (e.g. bradykinin/acetylcholine (ACh)/insulin/substance P) [64]. It is also recognised to exert important antioxidant, antiadhesive, and anti-proliferative effects [70]. NO diffuses freely through the vessel wall into platelets activating soluble GC to increase intracellular cGMP levels [71]. This in turn activates PKG leading to reduced intracellular calcium and inhibition of IP_3 which reduce cell activation leading to reduced platelet activity and vasodilatation. cGMP also inhibits the actions of phosphodiesterase (PDE) 3A who's role it is to breakdown cAMP [70].

1.5.3 Adenosine

Endothelial cells produce ectonucleotidases which are enzymes that dephosphorylate ADP to AMP and then to adenosine [72]. Adenosine acts through the platelet A_{2b} receptor to activate AC, leading to an increase in cAMP which reduces platelet aggregation [16]. Adenosine has also been shown to be an important regulator of blood vessel tone under hypoxic conditions [73].

1.5.4 The inhibitory effect of endothelial NO and PGI_2 on platelet reactivity

As outlined above, NO causes the production of cGMP while PGI_2 produces cAMP. Elevation of either cGMP or cAMP in platelets causes a reduction in platelet reactivity and the two together are strongly synergistic, as established more than 25 years ago [74], with platelets with elevated levels of cAMP and cGMP rendered rather unresponsive [75]. Because of the key roles of these critical secondary messengers in regulating cellular signalling and function, platelets are also equipped with a range of enzymes that rapidly remove both cAMP and cGMP, the PDE's which are also fundamental for platelet function [76]. A very dynamic balance exists in platelets with endothelial cell inhibitory mediators constantly stimulating the formation of cAMP and cGMP and intraplatelet systems constantly removing them, with the reactive state of platelets determined as a product of these two systems.

With this we can construct a view of how the endothelium promotes platelet inhibition and so assists in maintaining blood fluidity; platelets are central to blood clotting and their innate reactivity is balanced by a 50-fold excess of endothelial cells. These endothelial cells make intimate contact with the platelets principally in the microvasculature exposing them to NO and PGI_2 and so elevating platelet levels of cGMP and cAMP, which are strongly inhibitory and 'tame' platelets during their journey through the vasculature through multiple effects [77-79], including the regulation of platelet binding to fibrinogen [80] and collagen [81], stimulating calcium extrusion [82] and calcium uptake into the dense tubular system [83], inhibiting phospholipases [84], reducing platelet pro-thrombinase activity [85], decreasing thrombin binding [86], inhibiting P-selectin expression [87], inhibiting GPVI dimerization [88], phosphorylating GPIb inhibiting collagen-induced polymerization of platelet actin [89], reducing PS

exposure and blunting platelet pro-coagulant activity [90]. Therefore, fundamentally cyclic nucleotides are global inhibitors of platelet function and their production is dependent on NO and PGI_2 produced by the endothelium, once described as the maestro of the circulation by Sir John Vane. Importantly, but not often emphasized anti-platelet drugs interact with cyclic nucleotide systems in platelets and these are the same systems that mediate key endogenous pathways of platelet regulation. After considering the roles of the endothelium and platelets in atherothrombosis these anti-platelet drugs will be reviewed in detail and then these important pathways and interactions considered.

1.6 Atherothrombosis

Thrombosis describes the process of formation of a blood clot inside a blood vessel obstructing the flow of blood through the circulatory system. It is the same protective mechanisms involved in haemostasis that lead to the development of a pathological thrombus at inappropriate sites under certain conditions, which can be potentially detrimental to the host. Obstruction to blood flow can result in anoxia and oxygen deprivation leading to infarction and tissue death.

Thrombosis can occur in both the venous and arterial sides of the circulatory system. Thrombosis in the venous circulation is mainly dependent on coagulation factors. In the arterial side however, platelets are the main drivers with the physiological response potentially becoming a major cause of pathology. The processes involved in atherothrombosis are covered in the next section of this thesis.

Cardiovascular disease, involving the heart and vasculature is the leading cause of mortality and morbidity worldwide with one third of the population dying of complications attributable to atherothrombosis. 17.3 million deaths occur each year, mainly from ischaemic heart disease (IHD) and cerebrovascular disease, a number which is expected to grow to 23.6 million by 2030 [91]. Future cardiovascular burden is likely to be aggravated by the obesity epidemic, an aging population, the increasing prevalence of cardiovascular risk factors and insufficient implementation of prevention strategies [92].

1.6.1 Atherosclerosis

Atherothrombosis is a potentially devastating complication of atherosclerosis which is a chronic, inflammatory systemic disease leading to vascular events such as myocardial infarction (MI), cerebrovascular accident (CVA) or peripheral arterial disease (PAD). Mechanisms as to which specific sites are affected whilst others at distinct locations are spared are not fully understood but genetic predisposition, smoking, HTN, hypercholesterolaemia, DM and obesity are all recognised risk factors for the development of the disease.

The endothelium and the vascular wall constantly undergo repair processes following mechanical and chemical injury however, prolonged exposure to the above risk factors can impair these repair processes leading to inflammation and endothelial dysfunction [93]. Adherent platelets at sites of inflammation play a crucial role in the development of atherosclerosis by releasing cytokines, chemokines and growth factors promoting endothelial dysfunction and leukocyte recruitment [94]. There are two major initiators involved in the development of atherosclerosis, the production of ROS and lipid oxidation. Firstly, ROS is associated with a reduction in the anti-inflammatory and anti-atherogenic activities of NO which leads to endothelial dysfunction. ROS activate matrix metalloproteinases (MMP's) which can cause plaque destabilisation and rupture and also activate the transcription factor NF-κβ which initiates the expression of pro-inflammatory and pro-thrombotic genes. The second important factor is the oxidation of low-density lipoproteins (LDL) in blood vessels which leads to an inflammatory response and the development of atherosclerosis with foam cell formation. Foam cells will accumulate to lead to the apparition of fatty streaks which are the earliest atherosclerotic lesion to appear. With time, atherosclerotic plaques develop with their

pro-inflammatory milieu supporting accumulation of lipids, smooth muscle cells, monocytes, T cells and platelets leading to the apparition of mature plaques characterised by a complex necrotic lipid and cellular core covered by a fibrous cap [93]. These lesions can remain asymptomatic however, as plaque progression continues the fibrous cap can become eroded, thin and weak precipitating plaque disintegration and rupture exposing its highly thrombogenic core to circulating platelets [95]. This can lead to platelet activation, aggregation and thrombus formation, potentially presenting as a clinically relevant cardiovascular event. This is recognised as an acute coronary syndrome (ACS) should thrombus formation take place in a coronary artery.

1.6.2 ACS

Three types of ACS have been distinguished and described as occlusive, non-occlusive and disperse coronary thrombosis. Occlusive coronary thrombosis results in ST-segment elevation and Q-wave formation on the electrocardiogram (ECG) and is otherwise known as a STEMI (ST segment elevation myocardial infarction). Non-occlusive thrombosis is seen in NSTEMI (Non-ST segment elevation myocardial infarction) which can be associated with ST-segment depression, T wave inversion or a normal ECG. Dispersive coronary thrombosis represents unstable angina which can result in ischaemic changes on the ECG or present with a normal ECG but can also result in death due to arrhythmia. Interestingly, there is a circadian rhythm associated with thrombotic events which are known to occur with increased frequency in the morning. Reasons put for forward for this are increased platelet reactivity and hypercoagulable blood or higher levels of beta-adrenergic hormones.

These acute cardiovascular events are associated with high morbidity and mortality. Therefore, aggressive strategies such as lifestyle changes and therapeutic intervention with statins, angiotensin-converting-enzyme (ACE) -inhibitors and beta-blockers are prescribed as an effective preventative approach in reducing further events. Given the central role of platelets at each stage of atherothrombosis, anti-platelet agents that serve to inhibit specific steps in the platelet activation process are the mainstay of treatment for patients with established cardiovascular disease. A thorough overview of these different therapeutic agents is given in the next section. The mechanisms of action of two of the most commonly prescribed agents, aspirin and $P2Y_{12}$ receptor antagonists will then be related to the endogenous pathways in both endothelial cells and platelets that I have alluded to so far in this thesis.

1.7 Anti-platelet therapies

Several classes of agent are indicated in the treatment of cardiovascular disease, with substantial therapeutic developments having been made over the last decade. The principal therapeutic agents are reviewed below.

1.7.1 Aspirin

Acetylsalicyclic acid, first marketed in tablet form in 1899 by Bayer, is established in clinical practice as the default anti-platelet therapy in cardiovascular disease. Acetylsalicyclic acid is derived from the naturally occurring substance salicyclic acid, from Latin 'salix' for willow tree, the source from which it used to be obtained. Salicylates's anti-inflammatory, anti-pyretic and analgesic properties have been recognised for over 2000 years and were known to physicians such as Hippocrates and Galen. Aspirin was first synthesised in the late 19^{th} century when Hoffman acetylated the benzene ring of salicylic acid [96]. Aspirin exerts its therapeutic effects by preventing the formation and release of prostaglandins. The elucidation of the mechanism of action of aspirin came from the seminal work of Sir John Vane who in London in 1971 identified the shared mechanism of action of aspirin like drugs [97], work for which he shared the Nobel Prize in Physiology and Medicine in 1982 with Sune Bergström and Bengt Samuelsson.

As already mentioned, AA through the cyclooxygenase activity of PGHS (COX) is converted to PGG_2 which is further metabolised into PGH_2 though PGHS's peroxidase activity. From this PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 and TXA_2 are produced [98]. Aspirin acts by permanently inhibiting the COX enzymes which catalyse this first committed step to prostanoid synthesis. It causes irreversible inhibition of COX-1 through acetylation of

serine 529 residue and serine 516 residue in COX-2 causing a conformational change to the active site leading to inhibition of AA binding [99]. NSAIDS on the contrary cause reversible inhibition of COX by temporarily binding to the channel and obstructing the active site during the active period of the drug before it is metabolised [100]. Due to this shared mechanism of action and COX target it is thought that NSAIDs interact with aspirin reducing its overall anti-thrombotic properties [101]. Aspirin is also reported to have effects on haemostasis which are unrelated to its ability to inactivate COX-1, namely suppression of plasma coagulation and enhancement of fibrinolysis [102].

Aspirin is rapidly absorbed from the stomach and small intestine with peak plasma levels occurring 30-40 minutes post ingestion and evidence of platelet TXA_2 inhibition by 1 hour [103]. The oral bioavailablity of aspirin is 40-50%, although COX-1 is acetylated in the presystemic circulation so platelet inhibition is largely independent of this. Aspirin has a short half-life in plasma of 15-20 minutes so it is only detected for a short time before it is metabolised in the liver, gut and blood [104].

In platelets, by irreversibly blocking COX aspirin inhibits the production of TXA_2 [97, 98, 105]. TXA_2, when unchecked, drives further aggregation through stimulation of TP receptors on neighbouring platelets. While aspirin is short-lived in the circulation, it permanently inhibits the COX-1 enzyme through acetylation, and as platelets lack the apparatus to produce replacement COX-1 protein individual platelets remain inhibited for their lifetime [106]. Platelets circulate for around 7-10 days and so the concept has arisen of once a day dosing with aspirin to produce blockade of the entire platelet population in most individuals [107]. The human body turns over approximately 10% of platelets a day although this can be increased in certain diseases associated with high

platelet turnover such as DM. Since only a small proportion of uninhibited platelets have been shown to be required to trigger a thrombotic response a twice daily dosing regime has been suggested in these patient groups [108]. In contrast, inhibition of COX-2 requires much higher doses and more regular dosing intervals because high peroxidase tone at sites of inflammation inhibits COX-2 acetylation by aspirin and because nucleated cells rapidly resynthesize the enzyme [109].

Aspirin is currently the first line anti-platelet agent for the secondary prevention of CAD. This is based upon robust, widely accepted data that in patients with occlusive vascular disease low dose aspirin reduces thrombotic events by around 30% [110-112]. Aspirin is effective in doing this at what is generally referred to today as low dose, 75-100mg/day. Interestingly, this dose has evolved from 900-1500mg previously used in cardiovascular trials [113-115]. The second international study of infarct survival (ISIS-2) randomised controlled trial (RCT) published in 1988 actually analysed the safety and efficacy of low dose aspirin (162.5mg) at one-tenth of the aspirin dose employed in earlier trials and demonstrated a 23% reduction in mortality, a 46% reduction in non-fatal stroke and a 49% reduction in non-fatal reinfarction [113]. Several influential Antiplatelet Trialists' Collaboration meta-analyses have been conducted over the last three decades with the first in 1994 recommending an aspirin dose of 75-325mg as the most effective regime, which was later refined to 75-150mg in the 2002 Antithrombotic Trialists' Collaboration [114, 115].

The efficacy and safety of aspirin has been evaluated in a spectrum of clinical phenotypes from patients presenting with STEMI to healthy low-risk individuals. Many studies conducted consistently demonstrate the benefits of aspirin in the prevention of

thrombotic events in high risk patients where the benefits clearly outweigh the risks with a 4-8% annual thrombosis risk, whereby aspirin prevents 10-20 fatal and non-fatal vascular events per 1000 patients (NNT 50-100) at the cost of 1-2 major bleeding complications for 1000 per year (NNH 500-1000) [115]. Aspirin however, is not recommended for primary prevention in low risk individuals without pre-existing vascular disease. Although trials in primary prevention show a reduction in ischaemic events this is associated with an increase in bleeding, both in the GI tract and haemorrhagic stroke [116, 117]. It was estimated that aspirin in primary prevention reduced fatal MI 1-3 per 1000 however, was associated with 1-2 per 1000 major GI bleeds and 0.1-0.2 per 1000 intracranial bleeds [116]. Therefore, in these patients, decisions regarding anti-thrombotic therapy need to be made on an individual patient basis. This is because aspirin therapy is associated with an increased risk of haemorrhagic complications and it is generally accepted that aspirin-induced impairment of primary haemostasis cannot be separated from its antithrombotic effect [100]. This critical balance will depend on the assessment of absolute thrombotic risk versus the bleeding risk in an individual patient and it is important to evaluate the net benefit of aspirin therapy versus the harm in order to avoid potentially devastating complications such as intra or extracranial bleeds which, are thankfully rare complications. Aspirin can also cause serious GI bleeding as a result of direct inhibition of COX-1 in the gastric mucosa, as well as COX-1 inhibition in platelets. The incidence of GI complications however, appears to be related to the aspirin dose employed [100]. Although currently only recommended in guidelines for the prevention of cardiovascular disease aspirin therapy has been associated with a reduction in the incidence of cancer [118], especially colon cancers [119]. This chemopreventative action of aspirin and other NSAIDs has been linked to inhibition of both COX-1 and COX-2 [118].

1.7.2 $P2Y_{12}$ receptor antagonists

The secondary mediator ADP which is released from dense platelet granules following platelet activation plays a crucial role in thrombus formation and therefore, its receptors are valuable targets for anti-thrombotic therapies. As discussed, ADP mediates its effects through activation of two purinergic receptors on platelets: $P2Y_1$ and $P2Y_{12}$. The $P2Y_1$ receptor is expressed in virtually all tissues in the human body. Antagonists of the $P2Y_1$ receptor have the potential to be effective anti-platelet agents and are under development. The $P2Y_{12}$ receptor has a much more selective tissue distribution and as such has been an important target for anti-thrombotic agents for a number of years. $P2Y_{12}$ antagonists are currently prescribed alongside aspirin as dual anti-platelet therapy (DAPT) which is recommended for the secondary prevention of atherothrombotic events in patients with ACS or following percutaneous coronary intervention (PCI).

1.7.2.1 Thienopyridines

This class of drug irreversibly blocks the $P2Y_{12}$ receptor to inhibit the pro-aggregatory effects of ADP on platelets. Until recently, these agents included ticlopidine and clopidogrel but over the last decade prasugrel has also been developed. The reversible nonthienopyridine platelet ADP $P2Y_{12}$ inhibitors ticagrelor and cangrelor are also now in clinical use.

1.7.2.1.1 Ticlopidine

Ticlopidine, a pro-drug requiring a two-step metabolism by cytochrome P450 (CYP P450) enzymes became the first $P2Y_{12}$ receptor antagonist for clinical use in 1978 [120]. Despite reducing cardiovascular events by 30% its utility was hindered by GI side effects and toxicity which included neutropenia and rarely thrombotic thrombocytopenic

purpura (TTP) [121]. Following the results of a large meta-analysis which found compared to ticlopidine, its structural analogue clopidogrel significantly reduced major adverse cardiovascular events (MACE) (2.1% versus 4.0%, $p<0.002$) [120] and was not associated with adverse haematological side effects, ticlopidine was replaced by clopidogrel.

1.7.2.1.2 Clopidogrel

Clopidogrel is structurally related to ticlopidine but better tolerated with fewer adverse side effects. Like ticlopidine, it is inactive *in vitro* and requires a two-step metabolic conversion into its active compound to exert its anti-platelet effects. Following ingestion, 85% is inactivated by the CYP P450 enzyme, CYP2C19. The remaining 15% undergoes hydrolysis by the CYP3A4 enzyme into its active form which binds irreversibly to the platelet $P2Y_{12}$ receptor for the lifespan of the platelet. This prolonged metabolism accounts for the slow onset pharmacological profile of clopidogrel which takes 4-5 days to reach maximum effect of platelet ADP inhibition at a daily dose of 75mg, although this can be reduced to 2-5 hours following a loading dose of 300-600mg [122]. Clopidogrel has several other limitations despite being the workhorse of this class of drug for over a decade. Clopidogrel is associated with a wide individual variability in pharmacodynamic response, relating to factors affecting its generation into an active metabolite, such as loss-of-function polymorphisms of the CYP2C19 gene, meaning that a third of patients do not achieve a satisfactory level of platelet inhibition [123]. Hyporesponsiveness can also be attributed to other factors such as nonadherence, poor absorption or comorbidities [124]. Many drugs have been shown to interfere with the metabolism of clopidogrel with well-known drug interactions at the CYP level to statins, proton-pump inhibitors (PPIs) and calcium channel antagonists [125].

Several clinical trials have demonstrated the benefits of clopidogrel prescribed in combination with aspirin. Since then DAPT has been adopted as standard treatment following ACS or PCI [126, 127] and although the optimum duration of this therapy is disputed (6, 12 or 18 months, for example) it has indeed translated into improved patient outcomes [128]. Addition of clopidogrel to aspirin in patients with NSTEMI or unstable angina reduced MACE by 20% in the landmark CURE trial [129]. In the CURE-PCI substudy death rates, MI and target vessel revascularization (TVR) were improved with a 30% relative risk reduction (RRR) at 30 days [130]. The advantages of clopidogrel have also been demonstrated in STEMI patients in the COMMIT/CCS-2 study which, in 2005 found the composite endpoint of death, re-infarction or stroke was significantly reduced from 10.1% to 9.2% (p=0.002) without a significant increase in bleeding with clopidogrel [131]. The use of clopidogrel has not been found to be beneficial in primary prevention. High-risk patients without recent events in the CHARISMA trial in 2006 showed no significant reduction in MI, stroke or death (6.8% versus 7.3%, p=0.22) but a significant increase in global use of strategies to open occluded arteries (GUSTO) moderate bleeding (2.1% versus 1.3%, p=0.046) compared to those patients treated with aspirin alone [132].

The dosing regime of clopidogrel has been assessed and optimised in several clinical trials. Of note, the CREDO trial found a significant decrease of 26.9% RRR in MACE at one year in patients undergoing PCI who received a loading dose of clopidogrel 300mg compared to placebo [133]. The CURRENT/OASIS trial reported that a 7-day double-dose clopidogrel regimen led to a decrease in cardiovascular events and stent thrombosis (ST) in patients undergoing PCI for ACS compared to standard dose. This reduction however, came at the cost of a significant increase in bleeding [134].

An Interesting point is that because of the manner in which this therapeutic approach has evolved most large-scale RCT carried out over more than a decade to assess the efficacy of $P2Y_{12}$ inhibitors have nearly always been conducted in the presence of aspirin. A sparse exception was the CAPRIE trial, which was the first major clinical trial to test the safety and efficacy of clopidogrel compared to aspirin published in the Lancet in 1996 [135]. This study of 19,185 patients with symptomatic coronary, cerebral or PAD found clopidogrel to be superior to aspirin in risk reduction of cardiovascular events (5.32% versus 5.83%; 8.7% RRR with clopidogrel; $p<0.05$). At that point, clopidogrel was considerably more expensive than aspirin and therefore, did not replace aspirin therapy in the treatment of cardiovascular disease. In the MATCH trial of patients with recent transient ischaemic attack (TIA) or ischaemic stroke the addition of aspirin to clopidogrel led to a non-significant reduction in major vascular events (RRR 6.4%) but a significant increase in bleeding (1.3% to 2.6%) [136]. Therefore, importantly and unbelievably, RCT assessing the efficacy of clopidogrel without aspirin are lacking.

1.7.2.1.3 Prasugrel

More recently, prasugrel and ticagrelor have been marketed as third generation $P2Y_{12}$ receptor antagonists. Prasugrel is a pro-drug which irreversibly inhibits the platelet ADP $P2Y_{12}$ receptor. Compared to clopidogrel it has a faster onset of action, produces stronger and more consistent levels of $P2Y_{12}$ blockade and is associated with less interpatient variability. Prasugrel is metabolised in a two-step process, initially rapidly hydrolysed by esterases in the intestine, liver and plasma into thiolactone which then undergoes CYP 450 oxidation to form the active metabolite R-138727 [137]. Importantly, prasugrel does not substantially inhibit the CYP1A2-, CYP2C9-, CYP2C19-, CYP2D6-, or CYP3A-mediated metabolism of co-administered drugs [138]. Its active

metabolite appears in plasma within 15 minutes of dosing and peaks at around 30 minutes [139]. The median plasma half-life of the active metabolite is approximately 4 hours, and excretion is mainly urinary [140]. The landmark TRITON-TIMI 38 trial compared prasugrel to clopidogrel in 13,500 patients undergoing PCI. Prasugrel significantly reduced the primary endpoint of cardiovascular death, MI or stroke (9.9% versus 12.1%, $p<0.001$) however, this occurred at the expense of significantly increased rates of thrombolysis in myocardial infarction (TIMI) major and minor bleeding 5% versus 3.8%, $p=0.004$) [141]. The net clinical benefit favoured prasugrel (12.2% versus 13.9%) however, identified two groups of patients who did not benefit; those patients weighing less than 60kg and those aged over 75 and therefore the dose prescribed to these patients is reduced from 10mg to 5mg. One group, those with a prior history of CVA or TIA actually showed net harm from prasugrel (HR=1.54, $p=0.04$) and this therapy is therefore, contra-indicated in these patients.

1.7.2.2 Nonthienopyridines

These represent a new class of drug which target the $P2Y_{12}$ ADP receptor.

1.7.2.2.1 Ticagrelor

The first cyclopentyltriazolo-pyridine (CPTP) is oral ticagrelor which received U.S Food and Drug Administration (FDA) approval in 2011 for use in patients following ACS. Ticagrelor is direct acting, not requiring conversion to an active metabolite and therefore, results in a more predictable pharmacological profile. It does however, undergo principally hepatic metabolism via the CYP P450 pathway with generation of active metabolites which bind reversibly to the $P2Y_{12}$ receptor at an independent ligand site, non-competitively blocking ADP induced platelet aggregation [142]. Ticagrelor has

a rapid absorption with a peak concentration reached at 90-180 minutes and a half-life of 7 to 12 hours so needs to be administered twice daily [143]. In the ONSET/OFFSET study, Gurbel *et al* compared the effects of ticagrelor (180 mg LD, 90 mg bd MD) to clopidogrel (600 mg LD, 75 mg od MD) on inhibition of platelet aggregation (IPA) in 123 patients with stable CAD on aspirin [144]. The primary end point for onset, IPA at 2 hours after loading (20 μM ADP) was significantly greater for ticagrelor than for clopidogrel (88% versus 38%) and was significantly greater at 0.5, 1, 4, 8, and 24 hours after loading and also at 6 weeks ($p<0.0001$ for all). Ticagrelor was significantly faster in offset after drug discontinuation and on day 3 after the last dose IPA in the ticagrelor group was comparable to the IPA in the clopidogrel group at day 5. The pivotal multicentre, double blind phase III PLATO trial comparing ticagrelor to clopidogrel in 18,624 patients with an ACS clearly demonstrated improved cardiovascular outcomes, including a reduction in ischaemic events and all-cause mortality with ticagrelor as compared to clopidogrel with comparable rates of major bleeds [145], in contrast to prasugrel in the TRITON-TIMI 38 trial [141]. This trial ultimately led to FDA approval for the drug in 2011. Interestingly, in keeping with other studies, for example the DISPERSE-2 trial [146], PLATO found significantly higher rates of dyspnoea and ventricular pauses associated with ticagrelor therapy. There was also an intriguing incongruity referred to as the 'North American paradox' in the study, with the outcome of the trial indicating reduced efficacy of ticagrelor versus clopidogrel in North American patients. The reason for this geographical difference remains unproven and could just be a consequence of the vagaries of substudy analysis. However, Mahaffey *et al* analysed patient characteristics based on geographical regions and raised the possibility that the higher doses of aspirin used in the United States could play a role in the differential response to ticagrelor [147]. Whilst this could be simply attributable to statistical chance, it highlights the possibility

of the existence of a class effect of $P2Y_{12}$ blockers interacting with aspirin, as has been previously suggested by Leadbeater *et al* [148] and Warner *et al* [149]. This hypothesis is being explored in ongoing largescale RCT, namely the GLOBAL-LEADERS, https://clinicaltrials.gov/ct2/show/NCT01813435; and TWILIGHT trials, which will provide important new information. For now, the FDA currently recommend the use of aspirin at doses of less than 100mg, with doses exceeding this possibly reducing the effectiveness of ticagrelor.

1.7.2.2.2 Cangrelor

Cangrelor is another potent, short-acting novel inhibitor of ADP induced platelet aggregation belonging to the CPTP class of drugs, which received approval from the FDA in June 2015. Like ticagrelor, it is reversible as it does not lead to a structural change in the ADP receptor. However, unlike ticagrelor it is an ATP analogue which binds competitively to the $P2Y_{12}$ receptor and is only available for use through the intravenous (IV) route. It does not require conversion to an active metabolite and is metabolised through dephosphorylation pathways in plasma [150]. Once administered, it leads to almost immediate $P2Y_{12}$ blockade and due to its half-life of 3-6 minutes results in rapid reversal of anti-platelet effects after cessation of the infusion [151]. Initially, two large phase III trials, CHAMPION-PCI and CHAMPION-PLATFORM were abandoned due to poor interim results in mid-2009. The CHAMPION-PCI trial compared cangrelor to clopidogrel 600mg loading dose finding no significant difference in either the primary or secondary end points studied between the two groups [152]. The CHAMPION-PLATFORM study randomized patients with an ACS to receive either cangrelor or placebo during PCI. Again, the trial was terminated early as it was unlikely that it would show superiority of cangrelor. The primary end-point of composite death, MI or

ischaemia-driven revascularization at 48 hours post-PCI was non-significantly decreased by cangrelor (7.0% versus 8.0%, p=0.17). A reduction in the secondary end point of a reduction in ST rates was observed however, significantly higher bleeding rates were also observed in the cangrelor group [153]. The CHAMPION PHOENIX trial was a later phase III randomized study comparing cangrelor to clopidogrel in 11,145 patients undergoing PCI. Cangrelor reduced the likelihood of patients experiencing death, MI, ischemia-driven revascularization, or ST (5.9% to 4.7%), with the occurrence of ST reduced (1.4% to 0.8%)[154]. The BRIDGE trial evaluated the use of cangrelor as a bridge to coronary artery bypass graft (CABG) surgery in patients receiving DAPT. Results confirmed the efficacy and safety of cangrelor in this population demonstrating that cangrelor could represent an effective bridging therapy for patients taking thienopyridine antiplatelet agents, such as clopidogrel who are scheduled for surgery. Patients randomised to the cangrelor arm had significantly lower levels of platelet reactivity throughout the treatment period compared with placebo (98.8% versus 19.0%, p=0.01) but did not experience increased bleeding rates (11.8% versus 10.4%, p=0.76) [155].

1.7.2.2.3 Elinogrel

Elinogrel is a novel reversible ADP $P2Y_{12}$ receptor antagonist which can be given both orally and IV. It does not require activation and has a rapid onset and offset of action. Elinogrel was assessed in the phase II trial INNOVATE-PCI, which showed platelet inhibition was significantly higher with elinogrel compared with clopidogrel [156] but no differences in major or minor bleeding were observed [157]. There are currently however, no Phase III trials of elinogrel planned.

1.7.3 PDE inhibitors

Whilst aspirin and $P2Y_{12}$ receptor antagonists block specific receptors, PDE inhibitors reduce the breakdown of cyclic nucleotides. As discussed, cAMP and cGMP regulate platelets by activating PKA and PKG respectively, which phosphorylate intracellular protein substrates. Hence, the intensity of the signal transduction in platelets is determined by the concentration of these cyclic nucleotides with elevation of either causing a reduction in platelet reactivity [158]. Because of the key roles of these secondary messengers in regulating cellular signalling and function, platelets also contain a range of enzymes that rapidly degrade both cAMP and cGMP, the PDEs. These are fundamental for platelet function and are the targets of this class of drug. There are 11 isoforms of PDEs in the human body with different tissue distributions, specificities, targets and sensitivities to inhibitors [159]. There are over 100 PDE proteins and platelets are known to express PDE-2, PDE-3A and PDE-5 [160]. PDE-2 and PDE-3A hydrolyze cAMP and PDE5 hydrolyzes cGMP and therefore, both PDE-3A and PDE-5 inhibitors are marketed. It is important to note that PDEs are not specific to platelets but are widely expressed in various tissues and cells and PDE inhibitors therefore, produce a wide range of pharmacological effects and are associated with a broad range of side effects such as headache, palpitations, and diarrhoea.

1.7.3.1 Cilostazol

Cilostazol is a potent inhibitor of cAMP specific PDE-3A in platelets and smooth muscle cells where it increases levels of cAMP to produce smooth muscle relaxation and platelet inhibition [161]. Cilostazol inhibits platelet aggregation induced by various agonists. It is metabolised by CYP3A5 and CYP2C19 and is associated with substantial interindividual response [162]. Cilostazol was first introduced in 1988 and has been used for the

treatment of PAD and stroke. It was tested against placebo in the cilostazol stroke prevention study and found to be effective in the prevention of reoccurrence of stroke [163]. In a study evaluating the effects of cilostazol on walking distances in patients with intermittent claudication caused by PAD, cilostazol significantly increased absolute claudication distance at all measured time points [164]. The addition of cilostazol to DAPT has been shown to achieve greater platelet inhibition in patients with MI [165]. Cilostazol has been shown to prevent restenosis following PCI through inhibiting vascular smooth muscle cell proliferation. In the CREST and DECLARE-DIABETES trials, a reduction in stent restenosis was observed with the addition, of cilostazol to DAPT following BMS and DES implantation [166] [167].

1.7.3.2 Dipyridamole

Dipyridamole primarily blocks PDE-5 which hydrolyzes cGMP. In the European stroke prevention study 2 (ESPS-2), dipyridamole was tested in the treatment of TIA and stroke. It was found to be as effective as low-dose aspirin in the reduction of stroke and the combination of the two was twice as effective; aspirin 18%, dipyridamole 16% and the combination 37% compared to placebo [168]. The PRoFESS trial compared the efficacy and safety of two anti-platelet regimens, aspirin plus dipyridamole versus clopidogrel. The primary endpoint of recurrent stroke was 9% in the combination treated group and 8.8% in the clopidogrel treated group. The secondary outcome of stroke, MI, or death from vascular causes was 13.1% in both groups but there were more haemorrhagic events in the aspirin and dipyridamole group (4.1% versus 3.6%). This large non-inferiority trial concluded that there was no evidence that either of the two treatments was superior to the other in the prevention of recurrent stroke [169]. Today, dipyridamole has limited clinical indications and is only recommended in combination

with aspirin for people who have had an ischaemic stroke only if clopidogrel is contraindicated or not tolerated.

1.7.4 Glycoprotein IIb/IIIa inhibitors

The GPIIb/IIIa receptor is another target of anti-platelet therapy and the GPIIb/IIIa inhibitors represent the most potent platelet inhibitors in the treatment of acute thrombosis. As previously reviewed, although several different platelet agonists can stimulate platelet activation GPIIb/IIIa activation marks the final common step of platelet aggregation [170]. The importance of this receptor is highlighted in the rare autosomal recessive disorder Glanzmann thrombasthenia. These patients lack functional GPIIb/IIIa receptors and display impaired platelet aggregation to all platelet agonists but normal ristocetin aggregation and suffer mucocutaneous bleeding and increased bleeding following surgery or trauma [171].

There are three parental GPIIb/IIIa inhibitors currently approved for clinical use; the first to be developed was the monoclonal antibody abciximab, followed by eptifibatide and tirofiban which have shorter half-lives. These molecules bind to GPIIb/IIIa receptors in resting and active forms with different affinities in stimulated and unstimulated platelets. The binding site for abxicimab is on the β-chain of the GPIIb/IIIa receptor, which is different to the site of low molecular weight inhibitors eptifibatide and tirofiban which target the RGD ligand binding sequence. Abxicimab has a short plasma half-life of 10-30 minutes however, it has long pharmacodynamics due to high affinity and a slow dissociation rate from the GPIIb/IIIa receptor and thus, can remain bound to platelets for 15 days [172]. Tirofiban and eptifibatide bind reversibly to the GPIIb/IIIa receptor and have a plasma half-life of approximately 2 hours. Tirofiban is associated with a rapid

recovery of platelet reactivity within 4-8 hours of therapy cessation [173]. Cessation of eptifibitide therapy also results in early restoration of platelet activity [174].

GPIIb/IIIa inhibitors are indicated for use as adjuvant therapy in high risk patients undergoing PCI or in the treatment of unstable angina with planned PCI with an increased risk of thrombosis [175]. All three agents have been tested both upstream (administered after diagnosis) and downstream (given immediately before) of PCI.

1.7.4.1 Abciximab

Abciximab administered as a bolus before PCI followed by a 12 hour infusion in addition to unfractionated heparin and aspirin has been shown to reduce the risk of acute complications compared to placebo in three landmark trials; the EPIC, EPILOG and EPISTENT trials. Of note, theinopyridines were not administered to participants as they were not indicated at the time of the EPIC and EPISTENT trials. Abciximab in the EPIC trial significantly reduced the composite end point of death, MI or urgent intervention by 35% in high-risk patients undergoing PCI, although it did also lead to a doubling of bleeding complications [176]. The EPILOG trial also found a reduction in the composite endpoint of death, MI or urgent revascularization in more stable patients treated with abciximab and low dose-heparin (5.2%) and abciximab and standard-dose heparin (5.4%), as compared to placebo treatment (11.7%). Interestingly, in this study there were no significant differences in major bleeding among the treatment groups [177]. A similar reduction was observed in the EPISTENT study in the presence of theinopyridines, whereby abciximab treatment in combination with aspirin, ticlopidine and heparin was associated with a lower combined end point of death or MI compared to placebo in elective and urgent patients receiving a coronary stent (5.3% versus 10.8%)

or following balloon angioplasty (6.9%) [178]. More recently the ISAR-REACT study found the same rate of primary ischaemic outcome of 4% in elective patients receiving 600mg clopidogrel with or without the addition of abciximab [179]. However, the ISAR-REACT 2 trial which recruited patients with NSTE-ACS treated with aspirin and clopidogrel found that the addition of abciximab therapy reduced the composite outcome of death, MI or urgent revascularization (8.9%) versus placebo (11.9%) suggesting that this agent may be more effective used in the treatment of higher risk patients [180]. Results of studies in the context of STEMI however, have been mixed with some reporting the benefits of abciximab but others publishing negative results from the addition of this agent. Following the publication of the GUSTO IV-ACS trial, which showed not only no significant benefit from abciximab in medically treated patients but also a trend towards higher mortality associated with the administration of abciximab for 48 hours over placebo, this agent is not recommended for the treatment of patients in whom PCI is not planned and is also contraindicated in the upstream setting [181].

1.7.4.2 Eptifibatide

The effect of eptifibatide on platelet inhibition is dose dependent and the optimal dose was difficult to establish in initial trials such as the IMPACT [182] and PURSUIT [183] trials. This was thought to be due to the use of citrate as the choice of anticoagulant which led to an overestimation of the effect of eptifibatide following calcium chelation and the anticoagulant was changed to D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK). This, in conjunction with a dose increase to two 180 µg/kg boluses followed by a 2.0 µg/kg infusion led to levels of platelet inhibition of 90% in 90% of patients tested during PCI [184]. There were two large studies conducted with

eptifibatide in the downstream setting, namely the IMPACT-II and ESPRIT trials. In the IMPACT-II trial a comparatively lower dose of 135 μg/kg followed by an infusion of 0.5 μg/kg/minute led to a nonsignificant reduction in primary endpoint (9.2% vs 11.4%) [185]. The ESPIRIT trial employed a larger dose of two 180 μg/kg boluses and 2 μg/kg/minute in patients undergoing elective PCI. This trial observed a 37% reduction in primary endpoint of death, MI, urgent TVR and thrombotic bailout at 48 hours (6.6% vs 10.5%, p=0.0015) but also a significant increase in severe bleeding rates (1.3% vs 0.4%, p=0.027). The benefits of eptifibatide persisted at 30 days and 6 months [186]. However, eptifibatide, is not recommended in the upstream setting prior to visualisation of the coronary anatomy or decision to undergo PCI. Although results of the earlier PURSUIT trial indicated a reduction in death or MI at 30 days in the treatment group of NSTEMI patients managed medically or with PCI compared to placebo, these results were not mirrored in the later EARLY-ACS trial which investigated the early upstream use of eptifibatide compared to delayed in ACS patients treated either conservatively, by PCI or CABG. This strategy led to a significant increase in severe bleeding (2.6% vs 1.8%, p=0.02) but had no impact on the primary endpoint of all-cause mortality, MI, TVR or thrombotic bailout (9.3% vs 10.0%, p=0.23) [187].

1.7.4.3 Tirofiban

The RESTORE trial tested the use of downstream tirofiban compared to placebo in addition to aspirin and heparin in ACS patients undergoing PCI. A significant RRR of 27% in the primary endpoint of death, MI, repeat PCI or CABG due to PCI failure was observed at day 7 (p=0.02). However, this risk reduction became nonsignificant at day 30 (16%) and was lost at 6 months [188]. The PRISM and PRISM-PLUS studies investigated the use of upstream tirofiban in high risk ACS patients. The original PRISM study demonstrated

a 32% reduction of the composite endpoint of death, MI or refractory ischaemia at 48 hours in the group receiving tirofiban compared to the unfractionated heparin group [189]. However, the follow up PRISM-PLUS trial in which patients received either tirofiban and heparin or heparin or tirofiban alone was stopped prematurely due to excess mortality in the tirofiban treatment only group as compared to the heparin group only at 7 days (4.6% vs 1.1%) [190].

Importantly, many of the trials testing GPIIb/IIIa antagonists were conducted prior to the establishment of thienopyridines in the treatment of PCI patients and limited data have assessed the benefits of adding a GPIIb/IIIa inhibitor to the combination of aspirin and a $P2Y_{12}$ inhibitor. Although there is evidence that this class of agent can be of benefit in high risk patients treated with aspirin and clopidogrel undergoing PCI [191] the net beneficial effects are reduced and therefore, their use has become slightly marginalised partly due to the safer and cheaper treatment options of clopidogrel, prasugrel and ticagrelor. In those patients undergoing PCI receiving prasugrel or ticagrelor, GPIIb/IIIa inhibitors are restricted to bailout of thrombotic complications as no study has investigated the role of GPIIb/IIIa inhibitors in patients treated with these agents [126].

Drawbacks of this class of agent is their inability to inhibit platelet activation, associated thrombocytopenia and also their narrow therapeutic range because the same mechanisms are involved in haemostasis and thrombosis. Interestingly, oral GPIIb/IIIa antagonists are not clinically available. Orbofiban, sibrafiban, and xemilofiban have not been shown to be effective in reducing ischemic events following ACS [192]. In fact, they were found to increase mortality by approximately one-third [193].

1.7.5 PAR-1 antagonists

As previously discussed, thrombin exerts its effects on platelets through stimulation of PAR-1 and PAR-4 receptors. PAR receptors are abundant throughout the body and are found on many cell types and are involved in a number of processes including healing, neoplastic growth, vascular tone and inflammation, as well as of course, haemostasis and thrombosis [194]. Of the two PAR receptors found on platelets, PAR-1 is the target of this class of anti-thrombotic drug.

1.7.5.1 Vorapaxar

Vorapaxar is a novel, reversible oral anti-platelet agent which has been developed to competitively target the PAR-1 receptor. It is the first PAR-1 antagonist approved by the FDA for reduction of thrombotic events in patients with MI or PAD but is contraindicated in those with a previous stroke or TIA. Several phase II studies have taken place with vorapaxar with some finding that this agent was safe and helpful in reducing ischemic risk in patients. The TRA-PCI study investigated its use in patients undergoing non-urgent PCI already taking DAPT. This study found no increase in the primary endpoint of TIMI major or minor bleeding associated with vorapaxar therapy. They also found that the 60 day MACE rate was non-significantly reduced in those prescribed vorapaxar [195]. However, there have been studies which do not show any efficacy benefits but an increased risk of bleeding. The phase III trial TRACER randomized 12,944 ACS patients on DAPT to receive either vorapaxar or placebo. A finding of increased bleeding rates actually led to the recommendation that the trial terminate early however, the following endpoints had already been met. Vorapaxar therapy reduced the primary endpoint of a composite of death from cardiovascular causes, MI, CVA, recurrent ischaemia with hospitalization or urgent coronary revascularization (18.5% versus 19.9%, p=0.07)

however, statistical significance was not reached. The secondary endpoint of a composite of death from cardiovascular causes, MI or CVA was significantly reduced (14.7% versus 16.4%, p=0.02) however, this came at a cost of significantly increased GUSTO bleeding (7.2% versus 5.2%, p<0.001) [196]. The addition of vorapaxar to standard therapy was also assessed in 26,499 stable patients in the TRA 2 degrees P-TIMI 50 trial. Vorapaxar reduced the composite endpoint of cardiovascular death, MI or stroke as compared to placebo (9.3% versus 10.5%, p<0.001) however, vorapaxar similarly to the TRACER trial, was associated with an increase in GUSTO moderate or severe bleeding (4.2% versus 2.5%, p=0.001). Of concern, there was an increase in ICH in the vorapaxar arm (1% versus 0.5%, p<0.001) [197]. As a result, study investigators recommended premature cessation of vorapaxar in patients with prior stroke and it indeed remains contra-indicated in these patients. Scirica *et al*, on performing a pre-specified subgroup analysis of the TRA 2 degrees P-TIMI 50 trial suggested that vorapaxar could be helpful in those patients experiencing MI but without prior history of CVA [198]. A recent meta-analysis of 4 trials of vorapaxar concluded that adding vorapaxar to standard DAPT may be efficient in reducing the incidence of MACE but at the cost of increasing the risk of bleeding events [199]. Therefore, there are safety concerns, primarily bleeding associated with vorapaxar when prescribed in addition to standard DAPT. The role of vorapaxar may lie in patient populations in whom the benefits might outweigh the risks, such as those with PAD, a disease which is associated with a poorer prognosis following ACS.

1.7.6 Anti-coagulants

Early reperfusion therapy is standard in the treatment of STEMI. Fibrinolytic therapy with adjunctive unfractionated heparin to act against thrombin was easy to administer

and was shown to have marked benefits. This therapy has been largely superseded by primary PCI and adjuvant anti-platelet therapy and intravenous low molecular weight heparins (LMWH) such as fondaparinux which is superior to fibrinolysis [200].

1.7.6.1 Vitamin K antagonists

Several trials evaluating the role of oral anti-coagulants in AMI have taken place spanning over three decades [201]. Until recently the only available oral anticoagulants were the coumarin derivatives such as warfarin, which was first approved for use as a medication in 1954, having been initially introduced as a pesticide in 1948. The discovery of warfarin came as a result of the unexplained haemorrhagic deaths of cattle who had consumed mouldy silage made from a sweet clover plant, 'sweet clover disease' as it was known at the time. Chemist Dr Paul Link isolated the anticoagulant dicoumarol in 1939 as the cause of the disease. Named warfarin, after the Wisconsin Alumni Research Foundation (WARF) which funded his research, this agent went on to become the most commonly prescribed anti-coagulant for the next 60 years saving millions of lives. This class of agent, the so called vitamin K antagonists work by blocking the vitamin K-dependant production of coagulation factors II, VII, IX and X in the liver. Following the introduction of the International Normalised Ratio (INR) and lower doses of aspirin, warfarin therapy alone and in combination with the latter has been shown to reduce ischaemic events in several secondary prevention trials but at the cost of increased risk of major bleeding [202, 203]. Warfarin therapy however, has an established role in the treatment of venous thromboembolism (VTE) and also markedly reduces the risk of thromboembolism and stroke in atrial fibrillation (AF). However, more recently the rates of major and minor bleeding associated with triple therapy warfarin, aspirin and a thienopyridine have been highlighted as clinically important and significantly greater

than those reported with DAPT, leading to changes in the routine prescription of this therapeutic strategy and often the removal of aspirin from this regime [204].

One major drawback of warfarin is its narrow therapeutic window which requires monitoring to avoid life threatening bleeding although, adoption of the INR by the World Health Organization (WHO) in 1983 resulted in greater safety and effectiveness of the oral anticoagulant [201]. However, treatment with warfarin remains problematic despite monitoring, with less than two-thirds of patients found to be within the recommended INR range of 2-3 at testing, its many interactions with drugs and food types, and its most variable dose-response curve [205]. These limitations led to the development of the novel oral anti-coagulants (NOACs) in recent years.

1.7.6.2 Novel oral anticoagulants

The three currently licensed NOACs, rivaroxaban, dabigatran and apixaban are alternatives to vitamin K antagonists for use in the prevention of VTE in non-valvular AF, as well as for the treatment of deep vein thrombosis (DVT) and pulmonary embolism (PE). They are promising in that they have a predictable effect without the need for monitoring, fewer food and drug interactions, a shorter plasma half-life and an improved efficacy/safety ratio to warfarin [206]. Dabigatran is an oral direct thrombin inhibitor (DTI) and rivaroxaban and apixaban are oral factor Xa inhibitors.

1.7.6.2.1 Dabigatran

The FDA granted dabigatran exilate, the second clinically tested DTI approval in 2010 and issued a further statement in 2014 that compared to warfarin it reduced the overall risk of mortality and stroke but increased the risk of GI bleeding. It is a low molecular

weight peptidomimetic that binds directly and reversibly to the catalytic site of thrombin. It has low bioavailability, is a prodrug which is converted to the active drug dabigatran by serum and liver esterase hydrolysis with peak levels appearing at 2-3 hours. It has a half-life of 12 hours and excretion is predominantly via the renal route as unchanged drug [207]. In two large trials published in the New England Journal of Medicine (NEJM) the RE-LY trial and the RE-COVER trials, dabigatran was shown to be non-inferior to warfarin in patients with AF and VTE, respectively [208, 209]. It was also tested in a phase II study in secondary prevention following ACS in the RE-DEEM study in combination with aspirin and clopidogrel. Dabigatran patients showed higher bleeding event rates with limited reductions in ischaemic complications and dabigatran is therefore, not indicated in the ACS setting [210]. An antidote to dabigatran, idarucizumab, a monoclonal antibody, which completely reverses the anticoagulant effect of dabigatran within minutes has been developed by Boehringer Ingelheim and received approval by the FDA in October 2015 [211].

1.7.6.2.2 Oral factor Xa inhibitors

Factor X is synthesized by the liver. It is the first member of the final common coagulation pathway as it is positioned at the convergence point of the intrinsic and extrinsic pathways of coagulation. Factor X is activated by hydrolysis into factor Xa by both factor IX, with its cofactor factor VIII and factor VII, with its cofactor TF and goes on to catalyze the conversion of prothrombin to thrombin.

1.7.6.2.2.1 Rivaroxaban

Rivaroxaban is the first orally available factor Xa inhibitor, manufactured by Bayer. It is a highly selective reversible factor Xa inhibitor which inhibits prothrombinase and clot-

bound factor Xa. The compound is well absorbed from the gut with plasma levels peaking at around 4 hours. Its effects last 8–12 hours but factor Xa activity does not return to normal within 24 hours so rivaroxaban is prescribed once-daily. Two thirds is excreted unchanged in urine and the rest is metabolized in the liver by CYP P450 dependent and P450 independent mechanisms [212]. Importantly, is has no known drug-drug interactions. Rivaroxaban was shown to be more effective than enoxaparin in the prevention of VTE in the RECORD trials and as effective as warfarin in the treatment of VTE, PE and AF associated prevention of stroke in the EINSTEIN trials and ROCKET trials, respectively [213-215]. Its use was also evaluated in the secondary prevention of ACS in addition to aspirin with or without clopidogrel in the ATLAS and ATLAS-II studies. Although rivaroxaban reduced ischemic events it caused significant increases in major bleeding, clinically relevant bleeding, non-CABG bleeding and ICH in a dose-dependent manner and rivaroxaban is yet to receive FDA approval for use in the ACS setting [216, 217].

1.7.6.2.2.2 Apixaban

Apixaban, like rivaroxaban, is a highly effective reversible factor Xa inhibitor that inhibits prothrombinase activity and factor Xa within the prothrombinase complex. It has good bioavailability, reaches peak levels at 1-4 hours in plasma, has a half-life of 8-15 hours and is metabolized in the liver via CYP3A-4 dependent mechanisms. It was tested in the treatment and prophylaxis of DVT and PE in the ADVANCE and AMPLIFY trials and was approved for use in Europe in 2012 and in the US in 2014 [218, 219]. The ARISTOTLE trial in AF patients with a risk factor for stroke, found apixaban compared to warfarin therapy not only significantly reduced the occurrence of stroke and systemic embolism but also reduced the risk of major bleeding [220]. Apixaban however, is not indicated in the

secondary prevention of ACS after both APPRAISE trials showed increased dose-related rates of bleeding with little positive impact on ischemic events leading to their premature closure [221, 222].

From this overview, one can appreciate that all of these anti-platelet agents that act to reduce thrombotic risk are all associated with increased bleeding risk given the overlapping nature of the mechanisms of haemostasis and thrombosis. Given this, a wealth of cardiovascular research is ongoing with the ultimate goal of establishing therapies that will optimize thrombotic risk but not at the cost of bleeding. Agents in development at present include GPVI, GP1b, vWF and integrin $\alpha_2\beta_1$ inhibitors that are out of the scope of this review but hold future promise as potential anti-thrombotic agents.

Despite this broad range of therapeutic approaches recurrent coronary thrombosis still occurs. Given the central role of platelets in thrombosis there have been great efforts made to find *ex vivo* tests of platelet function that could assess and optimise on treatment platelet reactivity to improve patient outcomes. Some of the principle methods of platelet testing are reviewed in the next section after first highlighting the important interactions taking place between one of the most commonly prescribed anti-platelet therapies, DAPT and endogenous pathways in both platelets and endothelial cells. Key interactions that will become the principal focus of this thesis.

1.8 DAPT, NO and PGI_2

The aim of this next section is to highlight the key interactions taking place between the anti-platelet drugs, aspirin and $P2Y_{12}$ receptor blockers, prescribed as DAPT and endogenous platelet pathways whilst underlining the importance of the endothelium. For some time it has been appreciated that an important interplay exists between the endothelium, platelets and the mediators that they produce which, is fundamental to cardiovascular homeostasis. However, in more recent years exciting interplays between anti-platelet drugs and endogenous pathways in both platelets and endothelial cells are emerging.

1.8.1 Aspirin

As previously described platelets and endothelial cells use COX to produce TXA and PGI_2, respectively. Low dose aspirin prescribed today (<100 mg) is sufficient to completely inhibit platelets [107]. It has been demonstrated that lack of blockade of platelet COX-1 in people taking regular low dose aspirin is remarkably rare [223] and that in fact, in patients administration of 30mg daily is sufficient to suppress TXA_2 production by 95% [224]. Importantly, in addition to its effects upon platelets, aspirin will also inhibit COX at other sites in the body, including the blood vessel wall [225-227]. Higher doses of aspirin have anti-inflammatory, anti-analgesic and anti-pyretic effects which are mediated through inhibiting COX at sites other than the platelet. This can lead to changes in the balance of the formation of pro-aggregatory TXA_2 by platelets and anti-aggregatory PGI_2 by the blood vessel wall, a mechanism which is thought to account for the increased risk of thrombosis associated with strong, whole body blockade of COX enzymes [228]. These changes can be followed by the measurement of urinary metabolites [225, 226, 229-232]. For example, Fitzgerald *et al* established that 80mg

aspirin reduced urinary TXA_2 metabolites by around 80% and PGI_2 metabolites by around 50%; 325mg aspirin reduced the metabolites, respectively, by around 95% and 70% [225]. Interestingly, the investigators concluded that it was unlikely that any dose of aspirin could be completely selective for the inhibition of TXA_2 synthesis. In keeping with this, in 'at risk' patients with severe atherosclerotic disease even at a dose of 50mg aspirin significantly reduced urinary levels of PGI_2, as well as TXA_2 [233]. These findings along with similar reports by others [226, 234, 235] strongly suggest that aspirin even at low doses leads to substantial inhibition of COX within the vasculature, both in platelets and in the vascular endothelium and so reduces PGI_2 production [226, 236]. It is well recognised that reduction in PGI_2 signalling in platelets causes increased *in vivo* activation but it is also becoming clear that aspirin therapy could potentially have additional deleterious effects within the cardiovascular system by disrupting the powerful interplays between platelets, the endothelium and $P2Y_{12}$ receptor antagonists which, I describe below.

1.8.2 $P2Y_{12}$ receptor antagonists

As mentioned previously, NO and PGI_2 have long been recognised to synergise to produce powerful platelet inhibition [74]. Further key inhibitory relationships between $P2Y_{12}$ receptor antagonists and the endothelial mediators NO and PGI_2 have come to light in recent years. Importantly, in 2007 it was shown by Cattaneo *et al* that $P2Y_{12}$ receptor blockade potentiates the inhibitory effects of PGI_2 on platelets [237]. Furthermore, in 2013 Kirkby *et al* demonstrated that there also exists a powerful synergistic interaction between NO and $P2Y_{12}$ receptor blockade whereby, the blockade of $P2Y_{12}$ receptors dramatically increases the inhibitory effects of NO on platelets [238]. This is because the $P2Y_{12}$ receptor is a G-protein coupled receptor which is negatively

coupled to AC, a key regulator of cyclic nucleotides. $P2Y_{12}$ receptor activation blocks the action of AC rapidly turning off the production of cAMP and countering the inhibitory signalling actions of cGMP. When $P2Y_{12}$ receptors are blocked the effects of PGI_2 and NO acting through the cAMP and cGMP signalling pathways are unimpeded and so their inhibitory effects are notably increased relative to $P2Y_{12}$ receptor uninhibited platelets. PGI_2 and NO have synergistically inhibitory effects upon platelets and the interaction with $P2Y_{12}$ receptor blockers would theoretically provide a powerful three way synergistic effect: NO, PGI_2 and $P2Y_{12}$ receptor blockade are inhibitory individually, synergise with each other in individual pairs, and would synergise still further as a trio to provide far more potent platelet inhibition [239]. Thus, excitingly it could be that $P2Y_{12}$ receptor antagonists exert part of their powerful antithrombotic effect through potentiation of the endogenous, endothelial derived inhibitors of platelet function, NO and PGI_2. Accordingly, the important *in vivo* synergy between NO and PGI_2 may serve to amplify the anti-thrombotic effects of $P2Y_{12}$ inhibitors suggesting that the effectiveness of $P2Y_{12}$ receptor antagonists could vary in individual patients depending on their endogenous production of inhibitory mediators and that endothelial function is actually key in determining the therapeutic potential of $P2Y_{12}$ therapy. As a further layer of complexity, it should be noted that critically, a reduction in endothelial produced PGI_2 caused by the co-administration of aspirin could potentially hinder PGI_2's pivotal role in this powerful synergism. These vital concepts will be further investigated in this thesis following a brief review of current methods of both platelet and endothelial function testing.

1.9 Platelet function testing

Platelets have an established role in maintaining normal haemostasis and are therefore, associated with both bleeding disorders and thrombotic events. Traditionally, platelets were tested for evidence of bleeding disorders in specialist centres but today with our increased understanding of the core role of platelets in atherothrombosis, platelet tests are also performed which aim to identify patients at risk of cardiovascular events and to monitor anti-platelet therapies in non-specialist centres. The increasing number of patients taking anti-thrombotic therapies has led to the advent of simpler platelet tests which are applied to identify patients at risk of bleeding during surgery or post trauma and also in transfusion medicine.

The first *in vivo* platelet function test (PFT) to become established was the bleeding time assay by Duke in 1910. A cut would be made on the forearm and the time taken for cessation of bleeding recorded. More refined versions of this simple method remained established as the most reliable screening test until the 1990s. It has now been replaced by a screening bleeding history and more modern tests of platelet function as it is invasive, its reproducibility is low and it fails to identify subtle bleeding disorders. Essentially, it is not a useful predictor of the risk of bleeding in patients [240].

1.9.1 Light transmission aggregometry

In the 1960s, platelet testing was revolutionised by Gustav Born and John O'Brien who independently developed the concept of light transmission aggregometry (LTA) which remains the gold standard for assessment of various aspects of platelet functions in specialised laboratories today [241]. This test measures the ability of platelets in platelet rich plasma (PRP) to aggregate together following stimulation with an external agonist

in vitro. By adding a wide panel of agonists to PRP, a considerable amount of data can be obtained about the various pathways of platelet activation [242, 243]. The instrument consists of a light source and detector into which a cuvette containing PRP and a stirbar is placed. The test is based on detection of the increase in light transmission through the optically dense PRP with the precipitation of platelet aggregates following the addition of an exogenous platelet agonist. The aggregometer will record the rate and amount of maximal and final percentage aggregation (MA/FA) compared to the maximal optical density 0% (PRP) and minimal optical density 100%, platelet poor plasma (PPP).

This technique is associated with drawbacks. It may be affected by different pre-analytical conditions such as lipid plasma, haemolysis, or low platelet count. It requires relatively large volumes of blood, is labour intensive and time consuming, requiring skilled operators. Results may also be affected by procedural conditions such as PRP preparation and agonist concentration used. In an effort to improve and standardize the evaluation of platelet function, standardization processes and development of consensus guidelines on PFT have been developed, although these are more established in the bleeding rather than the thrombosis field [244]. LTA has been criticized for not representing a physiological test as platelets are not exposed to the endogenous factors that they would be *in vivo,* namely for example the endothelium, the extracellular matrix and the inhibitors PGI_2 and NO. It also creates a circular low shear system as opposed to the high parallel shear which is encountered in blood vessels and is a closed environment with high concentrations of single agonists which is not representative of the physiological environment. Nonetheless using LTA, high rates of residual platelet reactivity to ADP and AA have been associated with the development of ischaemic

events in patients with cardiovascular disease, and arbitrary clinical research cut-off values for ADP and AA-induced platelet aggregation have been suggested. However, LTA's predictive accuracy is only modest [245].

1.9.2 Modified 96-well plate assay

This technique is based on the same principles as LTA but is applied to a 96-well plate in order to test multiple platelet activation pathways [246, 247]. It is designed to be able to test multiple conditions at the same time, to reduce the labour intensity of LTA and also to reduce the amount of blood required. PRP and PPP are added to the plate to act as controls in a similar fashion to LTA. PRP is then added to incubation wells with agonists either coated or in solution, which will cause platelet aggregation upon stirring. The plate is typically stirred for 5 minutes before absorbance is measured in a conventional plate reader and represented as percentage aggregation.

1.9.3 Lumi-aggregometry

This technique is another modified assay based on LTA. It is essentially an assessment of platelet degranulation and secretion which contributes to the process of platelet activation. The release reaction is quantified by measuring the luminescence caused by the release of ATP, which is usually stored in dense granules [248]. This method is particularly useful in detecting disorders caused by storage pool and release disorders.

1.9.4 Whole blood aggregometry

Similarly to LTA, this method measures platelet aggregation however, rather than using optical detection it measures changes in impedance between two platinum wire electrodes as platelets aggregate in response to platelet agonists [249]. This is a useful

method as it bypasses the requirement for the separation of platelets from the rest of the blood which, is not only time consuming but removes other blood constituents which could affect platelet function [250]. However, the test is still time consuming and requires technical expertise.

1.9.5 Flow cytometry based methods

Flow cytometry is an extremely useful and resourceful technique for the study of platelets. It is used to measure platelet activation, to diagnose specific platelet disorders, measure reticulated platelets to monitor thrombopoiesis, monitor anti-platelet agents and count platelets, to name but a few of its applications. Washed platelets, PRP or whole blood can be utilised for flow cytometric assays with the latter having the advantage that platelets are assessed in their physiological milieu. This technique requires very small volumes of blood and provides very sensitive and specific characteristics of a large number of cells. However, disadvantages are that the equipment is expensive and the assay preparation is complicated requiring specialised training. Briefly, samples to be analysed are diluted to reduce platelet aggregate formation. Depending on the complexity of the experiment a minimum of two antibodies conjugated with two different fluorophores will be added; one acting as a 'platelet identifier' and the other recognising the antigen being tested. Following stimulation with a platelet agonist the samples are fixed and then assessed in the flow cytometer. Platelets can be recognised as they will exhibit the platelet identifier and characteristic light scatter.

Platelet activation is commonly measured through use of the activation-dependent monoclonal antibody PAC-1 and through antibodies specific to granule proteins such as

P-selectin. PAC1 is directed against the fibrinogen binding site which is exposed following a conformational change in GPIIb/IIIa receptors of activated platelets and therefore, only binds when the platelet is activated [251]. P-selectin is an adhesion molecule which is present on the membrane of α-granules in resting platelets. P-selectin is only expressed on the platelet surface following α-granule secretion and therefore, P-selectin specific monoclonal antibodies also only bind to activated platelets. P-selectin, via P-selectin glycoprotein ligand 1 (PSGL-1) mediates the adhesion of activated platelets to leukocytes. Flow cytometry can also be used to identify the interactions and binding of these cells to form multicellular aggregates as another marker of platelet activation.

It is not only extracellular markers of platelet activation that can be detected through flow cytometric analysis but also phosphorylation of specific intracellular platelet proteins that can be detected and quantified using phosphorylation-specific monoclonal antibodies. This is indeed possible as permeabilisation disrupts the outer platelet membrane permitting antibodies to bind to intracellular proteins [252]. One widely-studied example is phosphorylation of vasodilator-stimulated phosphoprotein (VASP). Under basal conditions VASP resides in a non-phosphorylated state. Prostaglandin E_1 however, though activation of cAMP leads to VASP phosphorylation. An action which is opposed by ADP binding to the $P2Y_{12}$ receptor but permitted by an active $P2Y_{12}$ receptor blocker. Therefore, the degree of VASP phosphorylation described as platelet reactivity index (PRI) will correlate with $P2Y_{12}$ activity and VASP represents a sensitive method to measure the effectiveness of $P2Y_{12}$ therapy [253].

1.9.6 Point of Care Analysis

Despite DAPT, some patients will still experience further thrombotic events questioning the appropriateness of the 'one size fits all' approach to antiplatelet therapy. Due to this and the labour intensive, specialist centre nature of the PFT described above, simple and quick tests that give clear results indicating the efficacy of antiplatelet therapy have been developed. This has led to the proliferation of point-of-care (POC) tests, defined as diagnostic testing at or near the site of patient care with the potential of identifying patients with high on-treatment platelet reactivity (HTPR) or those at risk of bleeding during surgery. This would potentially allow for superior risk stratification in order to then tailor anti-platelet treatment to achieve therapeutic levels with a view to improving patient outcome. The PFA-100, Plateletworks, Impact cone and plate(let) analyser are all examples of POC tests. VerifyNow and Multiplate are the most widely used POC tests, which have been applied worldwide in cardiovascular research studies but are not currently recommended for routine use in clinical practice in the UK. The VerifyNow is described below as an example of a POC test.

VerifyNow is a rapid, fully automated whole blood turbimetric-based assay, specifically developed to monitor the effects of antiplatelet drugs. It measures agonist induced agglutination of fibrinogen-coated beads by activated platelets expressing GPIIb/IIIa. Agonists used are AA, a combination of ADP and PGE_1 or TRAP to test for the effects of aspirin, $P2Y_{12}$ blockers and GPIIb/IIIa antagonists, respectively. The rate and extent of changes in light transmittance caused by the aggregating platelets is translated into $P2Y_{12}$ reactivity units (PRU) in the context of $P2Y_{12}$ antagonist therapy or aspirin reactivity units (ARU) and platelet aggregation units (PAU) respectively, for aspirin and GPIIb/IIIa cartridges [254]. In contrast to the more laborious "gold standard" in platelet

function testing, LTA, the ease of use of VerifyNow negates the need for skilled technicians and specialised laboratories and the results are not operator dependant [255]. It provides immediate information to facilitate prompt clinical decision making post PCI, for example. Importantly, it produces results which have been shown to correlate well with LTA [256] and in clinical trials such as ADAPT-DES, has provided biologically relevant results associated with adverse clinical outcomes such as ST [257]. Disadvantages include that it is inflexible and relatively expensive using disposable cartridges and only processing one sample at a time. It cannot be used for any other purpose than for monitoring the effects of three therapeutic agents. Factors such as platelet count, haematocrit, triglyceride and fibrinogen levels influence the performance of the assay, as does the time from blood sampling to testing [258], although this is true for most platelet function assays.

The clinical application of VerifyNow however, along with other POC tests is vastly debated worldwide as the additional value of PFT above the standard clinical predictors of poor prognosis commonly used in daily cardiology practice is only minimal [245]. Furthermore, no single POC test has been advocated or has conclusively demonstrated reliability and definitive utility to enhance clinical outcome [259]. Although the negative predictive value to predict bleeding during surgical procedures is high, the positive predictive value of each of these tests in atherothrombosis remains limited. In addition, distinct POC assays provide slightly different information on diverse platelet functions leading to variability in the reproducibility, accuracy and correlation of these tests and not all assays correlate well when compared to traditional tests of platelet function. There are other important arguments against their use. They represent a relatively crude reflection of cardiovascular activity and risk. They are fundamentally only a test of *in-*

vitro platelet function within a non-physiological environment and stimulus. They also only measure an isolated aspect of platelet function not reflecting the complicated nature of platelet physiology and the dynamic processes occurring in the vasculature. Furthermore, at present we cannot demonstrate that platelet reactivity *ex vivo* is a true representation of platelet activity *in vivo* and the precise HTPR thresholds that discriminate between patients with and without ischaemic events have not been determined. The next section will review the important clinical trials published in the area of PFT and clinical cardiovascular outcomes, before moving on to briefly describe tests of endothelial function.

1.10 Cardiovascular trials and personalised anti-platelet therapies

DAPT with aspirin and a $P2Y_{12}$ receptor antagonist is recommended for the secondary prevention of atherothrombotic events in patients with ACS or following PCI [127, 260, 261]. However, this anti-platelet therapy regime is associated with increased bleeding regardless of the choice of $P2Y_{12}$ inhibitor prescribed [129, 141, 145]. Clopidogrel has limitations which result in highly unpredictable $P2Y_{12}$ receptor inhibition. The reasons for this widely variable pharmacological response are several fold. The principal reason for this is that clopidogrel undergoes a two-step metabolism process, leading to the generation of variable active metabolite, which can result in HTPR in 1 in 3 patients treated with clopidogrel [262]. Age, ethnicity, DM, renal insufficiency, ACS, obesity and drug-drug interactions are all additional factors that contribute to diminished clopidogrel response. The main pharmacokinetic and pharmacodynamics factors contributing to this inter-individual variability however, are not completely recognised [263].

Ticagrelor and prasugrel provide more reliable and more potent $P2Y_{12}$ receptor inhibition and are effective in overcoming some of the limitations of clopidogrel treatment. Although recent studies have observed that HTPR can occur in response to prasugrel and ticagrelor treatment [144]. It has also been suggested that there may exist a ceiling effect even with these newer agents, as despite therapy there remains a 10% residual ischaemic event rate [264]. The pharmacodynamic benefits of these new agents have however, translated into significant clinical benefit but at a cost of increased bleeding. Optimal management of patients would ideally involve reduction of thrombotic and bleeding risks associated with $P2Y_{12}$ therapy to balance efficacy and

safety and reduce adverse outcome. It had been hoped that monitoring platelet reactivity would improve patient survival by providing a valuable option to enable the risk stratification of patients according to their bleeding and thrombotic risk. As described in the previous chapter, there are several devices which test platelet reactivity during $P2Y_{12}$ therapy. Unfortunately, this has not been the case and PFT has not been shown to be successful in reducing ischaemic complications and receives a class IIb recommendation in patients at high risk of poor clinical outcome where testing may alter management and a class III recommendation for routine use [265]. Therefore, currently despite this variability a one size fits all approach is still employed to DAPT in clinical practice. This is unfortunate as the concept of a therapeutic window of platelet reactivity is an attractive one, full of potential to improve patient outcomes in a similar manner to the INR utilised for warfarin.

Despite this, HTPR is a firmly established independent risk factor for thrombosis [266, 267] and there is accumulating evidence underscoring the importance of low on treatment platelet reactivity (LTPR) as a predictor of bleeding risk [268]. Several observational and larger outcome studies in patients undergoing PCI have reported HTPR to be linked to negative post-PCI thrombotic occurrences, with the strongest association demonstrating the link between short term outcomes post PCI, notably ST. These studies have been conducted employing various recognised laboratory methods, including LTA [269, 270], VerifyNow [245, 271], MEA [272] and VASP phosphorylation [273]. A meta-analysis also demonstrated the increased risk of cardiovascular mortality associated with clopidogrel non-responsiveness [267]. The prospective registry ADAPT-DES, employing a PRU cut-off value of 208 also highlighted the importance of HTPR as a risk factor for ischaemic events, for example ST and MI at 1 year ($p=0.005$ and $p=0.001$,

respectively) [257]. In contrast, in non-PCI patients the association is less clear. The use of PFT to assess drug responsiveness in the ADRIE study in medically managed patients with stable CAD did not add any incremental predictive value of thrombotic events over conventional risk factors used in routine clinical practice [274]. Although, it is important to note that these clinical risk stratification scores are sometimes of limited use as many of the risk markers for thrombosis such as age, HTN and renal failure are also applicable as predictors of bleeding so do not discriminate between the two opposite ends of the spectrum outcomes.

Despite the evidence linking HTPR to negative cardiovascular outcome, multiple large-scale prospective, RCT have all failed to modify overall thrombotic risk by tailoring antiplatelet therapy to reduce platelet reactivity and improve patient outcomes. Although, it has been repeatedly cited that all of these individual trials were fraught with major limitations.

The GRAVITAS study randomly assigned 2200 PCI patients with a PRU > 235 to receive either standard (300 mg LD, 75 mg MD) or high dose clopidogrel (600 mg LD, 150 mg MD) as treatment intervention based on a measure of platelet function to reduce post PCI thrombotic events. High dose clopidogrel treatment was ineffective in reducing composite ischaemic event occurrence [275].

The ARCTIC triallists randomly assigned PCI patients to either a strategy of platelet function monitoring or to conventional therapy to establish whether the former strategy was effective in improving clinical outcomes. In poor responders, therapy was intensified to high dose clopidogrel or prasugrel. However, no improvements were

observed in the platelet function monitoring group as compared to the standard therapy group [276].

TRIGGER-PCI sought to determine whether a strategy intervention of high dose clopidogrel based on a measurement of platelet function in PCI patients with HTPR optimised clinical outcomes. Unfortunately, the trial was pre-terminated due to lower than expected incidence of the primary endpoint of composite of ischaemic events [277].

Lastly, the TRILOGY trial conducted in medically treated ACS patients did demonstrate greater platelet inhibition by treatment intensification with prasugrel. However, not only did this not translate into event-free survival but the trial failed to show any firm link between HTPR and negative outcomes [278]. All of these trials put into question the use and prognostic relevance of PFT in patients undergoing PCI and therefore, it remains a vastly debated topic worldwide.

There are several pertinent issues concerning the use of tests analysing platelet function which include the lack of standardization of some methods, notably LTA. Published cut-offs for high and low platelet reactivity are highly heterogeneous and usually not validated outside of individual exploratory studies which, further fuels the controversy on the prognostic relevance of PFT in patients undergoing PCI. This means that currently there are no clinically validated thresholds distinguishing optimal platelet reactivity to enable precise risk stratification of patients following PCI. There are however, consensus-defined, uniform cut-offs for standardized platelet function assays which are based on exploratory studies and the best available evidence for each testing method.

These were reviewed in a recent collaborative analysis in 2015 on the role of platelet reactivity for risk stratification after PCI [279] where the following cut-offs were suggested for the VerifyNow, Multiplate analyser and VASP assay. LTPR-OTPR (Optimal on treatment platelet reactivity)-HTPR were defined as <95, 95-208, >208 PRU for VerifyNow, <19, 19-46, >46 U for Multiplate and <16, 16-50 and >50 for VASP assay. 20,839 patients from 27 studies were included in the analysis which found 41% patients to have HTPR, 20% LTPR and 39% OTPR. Patients with HTPR when compared to OTPR were at higher risk of ST [RR:2.73 (2.03-3.69), p<0.00001] and had a reduced bleeding risk [RR:0.84 (0.71-0.99), p=0.04]. Those with LTPR in contrast, had a higher bleeding risk [RR:1.74 (1.47-2.06), p<0.00001] but no elevation in ST [RR:1.06 (0.68-1.65), p=0.78]. Interestingly, HTPR was linked with significantly higher mortality (p<0.05). Of note, the use of LTA was discouraged unless the tests above were not available, due to its lack of standardisation.

Therefore, crucially no convincing relationship between the results of platelet testing and thrombotic events has been firmly established. Although there have been a few smaller scale studies that suggest that PFT and a therapy adjusted approach to PCI patient care could potentially be effective. Angiolillo *et al* demonstrated that a maintenance dose regimen of 150mg clopidogrel is associated with reduced platelet reactivity and enhanced platelet inhibition compared to that achieved with the 75mg in patients undergoing elective PCI [280]. Switching from clopidogrel to prasugrel has been shown to reduce HTPR [281] and also improved outcomes in the MADONNA study [272]. Bonello *et al* utilised VASP-P to tailor incremental loading doses of clopidogrel to below a set HTPR value to show significant reductions in ST and MACE [282]. In addition, a meta-analysis of 4213 patients by Aradi *et al* showed that the use of an intensification

of antiplatelet therapy protocol was associated with a significant reduction in cardiovascular mortality, ST and MI compared to conventional approach [283].

Given these positive results, trials investigating the benefits of personalised therapy are ongoing and include TAILOR-PCI, ANTARCTIC and GIANT, the results of which are eagerly awaited. The trial design of these studies have been carefully optimised as previous large scale RCT have been criticized and some believe that this would have impacted on the negative results. It was found in the ARCTIC trial that amongst the poor responders only 3.2% received prasugrel whereas 80.2% received high dose clopidogrel (600mg LD, 150mg MD). In the GRAVITAS trial 40% of low responders remained so despite the increased dose of clopidogrel. Patient sample sizes have been questioned with some suggesting that many more, at least 17,000 would be required to show statistical differences in event rates [284]. Patient selection has been condemned with the exclusion of high-risk ACS patients and predominant inclusion of stable and elective PCI patients as it is suggested that intensified anti-platelet therapy might improve outcomes particularly in high risk patients but may not be so effective in low risk patients. There are also debates around study endpoints and the time of testing and randomisation. Also thought to be important is the course of intervention to reduce HTPR with third generation $P2Y_{12}$ receptor antagonists now preferred to higher doses of clopidogrel. When considering alternative potential reasons for the failure of these studies, theories include that HTPR is a reliable method of risk stratification but not a variable to modify therapy [285]. That platelet reactivity is not solely a measure of drug response but also reflects the co-morbidities of the patient and PFT could be a poor substitute for the complex interactions taking place *in vivo* [286]. To this end, it has been suggested that

the addition of clinical variables and genotype to PFT could enhance risk prediction [287].

An alternative theory and the one investigated in this thesis is that the weakness of these trials and PFT could be that they do not take into account the powerful interactions described in this thesis between anti-platelet therapies, endogenous platelet systems and the endothelium, specifically the strongly synergistic relationship between $P2Y_{12}$ blockade, PGI_2 and NO. This could potentially limit their interpretation of true *in vivo* platelet function, which would be better represented as a product of intrinsic platelet reactivity and endothelial function. This thesis will go on to highlight the importance of endothelial driven *in vivo* modulation of $P2Y_{12}$ inhibition and introduce the concept of refining *ex vivo* PFT by incorporating an assessment of endothelial function to potentially better predict thrombotic outcomes in individual patients. Prior to this, the last section of this introduction comprises a short review of the tests available to assess endothelial function, some of which will be utilized in this thesis to start to test my hypothesis.

1.11 Endothelial function testing

As reviewed above, the endothelium plays a pivotal role in cardiovascular haemostasis and disease. It regulates vascular tone and inflammation and its integrity is critical to prevent circulating platelets from being exposed to subendothelial matrix proteins to prevent arterial thrombosis. Cardiovascular risk factors such as DM and HTN lead to an excessive production of superoxide which leads to NO inactivation. This loss of NO bioavailabilty proceeds the development of atherosclerosis and is associated with morbidity and mortality [288]. Endothelial dysfunction is a well-established risk factor for cardiovascular disease characterized by an imbalance between vasodilatating and vasoconstricting substances [289]. It is a feature of atherosclerosis and proceeds it when only risk factors are present.

Although the vascular endothelium has many functions the term 'endothelial function' is, in most cases, used to refer to the ability of the endothelium to release compounds that induce direct relaxation of smooth muscle cells within the vascular wall [290]. This is thought to largely reflect the bioavailability of NO but can also be representative of the bioavailability of PGI_2 and other EDHFs. Therefore, reduced activity of NO and other EDHF's can be detected through the assessment of endothelial function in a given individual. This is because endothelial dysfunction represents a syndrome with various clinical presentations rather than a localized vascular disorder [291]. This systemic nature has been demonstrated by the similar power of coronary and peripheral dysfunction in predicting cardiovascular events and that cardiovascular events can occur remotely from the site of dysfunction [292]. Assessment of the endothelium because of its location was previously limited. However, there are now several non-invasive, cost-

effective and reproducible techniques that can be employed to assess endothelial function to help evaluate cardiovascular risk. These tests rely on two main principles; that endothelial dysfunction is a systemic disease which can be measured in different vascular beds and that certain stimuli will lead to NO release from the vascular endothelium to produce vasodilatation. This section describes the invasive and non-invasive evaluation of endothelial function which are generally assessed by measuring vasomotion of conduit vessels or regional increases in blood flow, reflecting microvascular/resistance vessel function in the coronary or peripheral circulation [293].

1.11.1 Invasive tests of endothelial function

The gold standard in the assessment of endothelial function is performed in the coronary circulation at coronary angiogram with intra-coronary Doppler for visualisation of the direct calculation of blood flow changes in vessel diameter and resistance. However, this technique is invasive and complex requiring expensive equipment and specialised expertise which limit its use although it is widely used in tertiary centres.

Pharmacological stimuli such as ACh, serotonin or papaverine are delivered in increasing concentrations to assess epicardial coronary vasodilation. ACh is widely used as it is short acting and in atherosclerotic vessels it produces paradoxical vasoconstriction due to impaired muscarinic vasodilatation as a consequence of a poorly functioning endothelium, as reported by Ludmer *et al* in 1986 [294]. Adenosine and glycerlyl trinitrate (GTN) or sodium nitroprusside (SNP) are used to assess endothelium-independent vasodilation in the microcirculation and compared to that due to the direct relaxation of smooth muscle cells, respectively. Coronary artery endothelial dysfunction has been shown to independently predict acute cardiovascular events in patients [295].

1.11.2 Non-invasive tests of endothelial function

Similar principles to those applied to invasive testing are applicable to non-invasive endothelial testing. Healthy arteries will vasodilate in response to a pharmacological stimulus such as ACh or salbutamol (endothelium-dependent vasodilators) or reactive hyperaemia (RH) via NO release, as well as the release of other EDRFs [296]. Venous occlusion plethysmography (VOP) was developed over 100 years ago and is the oldest method to assess endothelial function. More commonly used techniques nowadays include ultrasound flow mediated dilatation (FMD) and pulse wave analysis (PWA) with inhaled salbutamol. Laser Doppler skin flowmetry (LDF) and digital pulse amplitude tonometry (PAT) are also used and focus on microvascular flow.

1.11.2.1 ***Venous occlusion plethysmography***

Strain-gauge plethysmography (SGP), first described in 1951 is the most widely applied method of VOP which represents a non-invasive technique assessing peripheral blood volume. Tissue blood flow is measured by assessing tissue volume change following inflation of a proximal cuff to a pressure which occludes venous outflow but not arterial inflow leading to a volume change proportional to arterial inflow. Local infusion into the brachial artery allows for a minimally invasive additional assessment of the direct effect of drugs such as ACh on vascular tone. These agents are administered in escalating doses to cause local forearm vasodilatation but not systemic hypotension. Responses to these endothelial agonists are compared to those induced by direct smooth muscle relaxing agents in order to evaluate endothelium dependent vasodilatation. This technique is reproducible and has been widely used in research but its invasive nature does limit its use relative to more modern non-invasive techniques.

1.11.2.2 Flow mediated dilatation

This technique involves the induction of a shear stress stimulus in the conduit brachial artery following a period of ischaemia induced by occlusion of forearm vessels for 5 minutes [297]. It measures changes in conduit vessel diameter by ultrasound, changes which occur with increases in blood flow following dilatation of the microvasculature. These increases in vessel diameter occur through endothelium dependent mechanisms [298] which reflect endothelial-derived NO bioavailability [299]. The brachial artery is most often used with a blood pressure cuff placed 1-2 cm above the antecubital fossa inflated to suprasystolic pressure. When released, RH results. The artery is measured at the end of diastole to assess the vessel response to increased flow which is expressed as percentage change from vessel baseline. FMD has been shown to be associated with cardiovascular events in patients with and without overt cardiovascular disease [300, 301] and also to the presence of cardiovascular risk factors.

1.11.2.3 Pulse wave analysis and pulse wave velocity

Arterial stiffness depends partly on smooth muscle tone [302]. Predominantly NO but also ET-1 and natriuretic peptides are known to contribute to the functional regulation of artery stiffness [303]. Analysis of the arterial waveform and velocity provides information about stiffness in arteries and the amount of wave reflection in the arterial system [303]. This can be assessed noninvasively by using PWA. Wave reflection occurs at sites of impedance mismatch usually occurring at branch points within the cardiovascular system. It is quantified using augmentation index (Aix) by determining the difference between at least two consecutive systolic peaks which, will decrease with vasodilatation and increase with vasoconstriction [304]. Increased arterial stiffness increases the velocity of both forward arterial blood flow, as well as reflected waves

causing central pressure augmentation [305]. It is possible by recording the shape of the arterial waveform following the administration of GTN (an endothelium independent stimulus) and after salbutamol (an endothelium dependent stimulus) to make an assessment of endothelial function [306]. PWV is another classic index of aortic stiffness which has been related to vessel dispensability. Aortic PWV is measured between the carotid and femoral arteries by synchronically detecting arrival of the wave in these two areas and measuring the distance between them. It is easy to measure non-invasively and is highly reproducible [307]. PWV has a strong correlation with cardiovascular events and has been shown to be an independent predictor of all-cause and cardiovascular mortality [308, 309]. PWA has been used to identify endothelial dysfunction in a variety of disorders including PAD where salbutamol-induced changes in Aix were significantly reduced in PAD patients while GTN-produced changes were not different [310]. Aix has also been found to correlate with traditional CAD risk factors and atherosclerosis [311].

1.11.2.4 Peripheral artery tonometry

This technique uses a finger plethysmography cuff which records finger arterial pulsatile volume changes. The EndoPAT device assesses post-ischaemic vasodilator response compared to baseline flow to assess endothelial function as the PAT signal is modulated by the bioavailability of NO [312]. A finger on each hand is monitored and a pressure cuff placed on one of the upper arms to allow for a hyperaemic response to take place with the other finger acting as a control. The EndoScore is calculated as a ratio of the PAT signal over the baseline signal. Endothelial assessments with PAT have been shown to demonstrate patterns of abnormality similar to that of brachial artery ultrasound assessment of FMD [313]. Digital vasodilator function was related to multiple traditional

and metabolic cardiovascular risk factors such as male sex, body mass index (BMI), ratio of total to high-density lipoprotein (HDL) cholesterol, DM and smoking in a Framingham heart study of third generation cohort participants [314]. Low RH signals, indicating endothelial dysfunction detected by EndoPAT have also been associated with higher adverse event rates during follow-up and provided incremental value to the Framingham risk score [315].

1.11.2.5 Laser Doppler flowmetry

LDF is a technique which monitors skin microvascular blood flow. This is because the response observed in the cutaneous circulation is thought to mirror the responses taking place in other vascular beds [316]. In contrast to the techniques detailed above it provides an assessment of the microvasculature. The laser light beam will change when it comes into contact with moving tissues. The fraction and magnitude of light shifted is dependent on the concentration of moving red blood cells and on their average velocity, respectively [317].

The direct delivery of ACh and SNP through iontophoresis and post-occlusive reactive hyperaemia (PORH) are techniques which are associated with LDF for the non-invasive measurement of endothelial function. RH will lead to an increase in both the concentration and velocity of red bloods cells following the release of a pneumatic cuff inflated to supra-systolic blood pressure reflecting NO and prostanoid availability. Iontophoresis is a technique describing the migration of charged substances through the skin by means of delivering a small continuous galvanic current. This allows very small amounts of drugs to be administered non-invasively. ACh can be used to induce vasodilatation reflecting NO and prostaglandin participation and SNP to test smooth

muscle dependent pathways of vasodilation [318]. LDF assessed ACh iontophoresis is impaired in several pathologies linked to cardiovascular disease including hypertension and hypercholesterolaemia [319].

1.11.3 Novel assays

In recent years novel diagnostic tools for assaying endothelial function have been developed. These include ELISA-based quantification of circulating biomarkers such as vWF, ET-1, t-PA, thrombomodulin, soluble VCAM-1 and ICAM-1 and E-selectin. Techniques evaluating and phenotyping circulating endothelial cells and endothelial-derived microparticles are also being tested. These remain very much investigational methods of endothelial function testing at present.

1.12 Summary

An often explored hypothesis is that in at risk patients the chances of experiencing a thrombotic event is associated with the level of platelet blockade: i.e. those individuals with less effective blockade provided by aspirin and, particularly, $P2Y_{12}$ receptor blockers are more at risk of thrombotic events. However, studies have failed to show any benefits from *ex vivo* monitoring of platelet function and subsequent tailoring of treatment in patients receiving DAPT. This failure is possibly because the *ex vivo* platelet tests used in these trials do not consider the environment in which platelets reside *in vivo*. Namely, that within the circulation endothelium-derived autacoids NO and PGI_2 reduce platelet reactivity and prevent inappropriate platelet activation. Taking account of the above observations, I hypothesise that within the circulation the levels of endothelium-derived mediators are an important determinant of the efficacy of DAPT. The strong synergies between $P2Y_{12}$ inhibitors and the cAMP and cGMP signalling systems mean that the *in vivo* platelet reactivity in patients receiving DAPT will be a function of the level of $P2Y_{12}$ receptor blockade and the levels of endothelial-derived NO and PGI_2. This provides an explanation for different thrombotic outcomes in the presence of similar levels of platelet blockade; i.e. individual patients with different levels of endothelial function, or indeed disease-driven endothelial dysfunction, would have different levels of *in vivo* platelet inhibition for the same level of DAPT activity, as determined by *ex vivo* testing and why even allowing for differences dependent upon adherence to therapy, individuals on DAPT may in fact present rather a more homogenous level of platelet inhibition than can be associated to different clinical outcomes. A schematic diagram of the ideas so far put forward in this thesis is represented in figure 1.3.

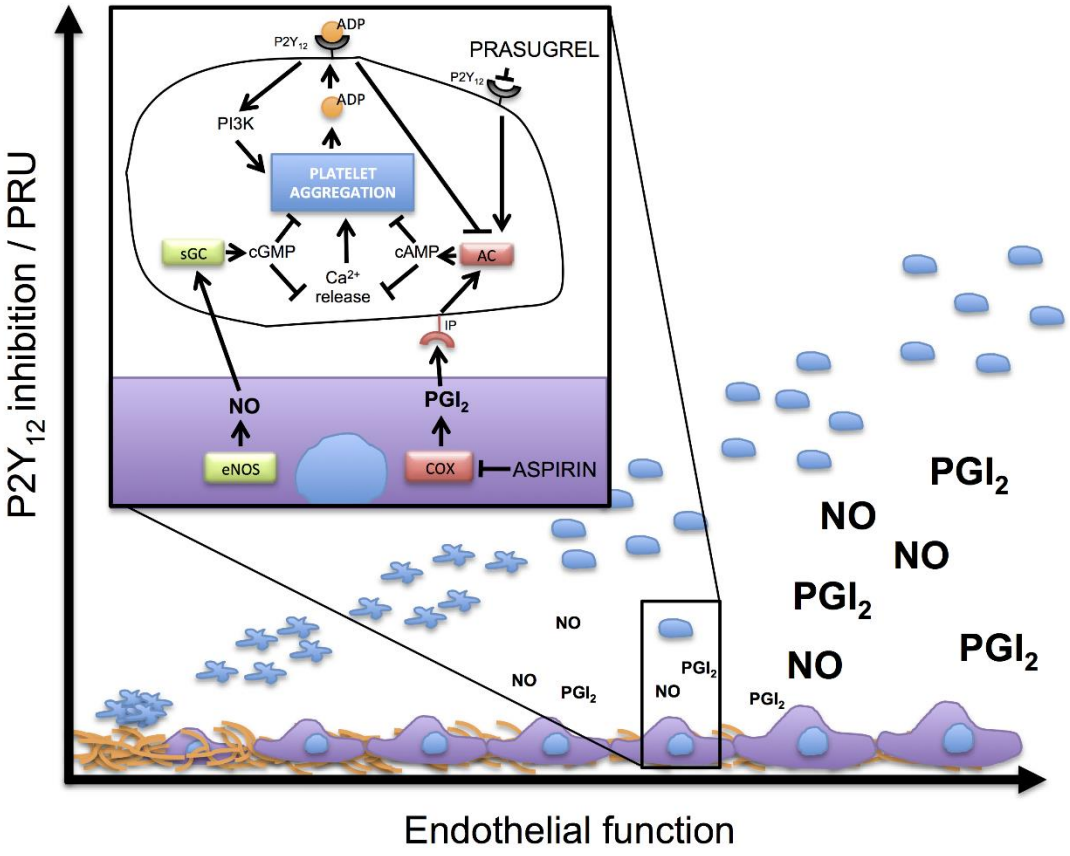


Figure 1.3: Summary of the interactions between the endothelium and $P2Y_{12}$ antagonism

1.13 Aims

This thesis investigates the complex interplay between $P2Y_{12}$ receptor blockade and the endothelial inhibitors NO and PGI_2.

This approach is structured into the following specific aims:

1. To investigate *in vitro* the interaction between $P2Y_{12}$ blockade, NO and PGI_2.
2. To demonstrate the anti-platelet effects of NO and PGI_2 in the presence of strong $P2Y_{12}$ receptor blockade in healthy volunteers *ex vivo*.
3. To consider the effects of cardiovascular disease on the relationship between NO, PGI_2 and $P2Y_{12}$ blockade in patients with PAD.
4. To develop a reproducible method of endothelial function testing in healthy volunteers in order to start to consider the role of the endothelium in platelet reactivity in patients.
5. To research the respective roles of platelets and the coagulation system in thrombus formation and the effects of aspirin, $P2Y_{12}$ antagonist and DAPT therapies.

Chapter 2: Materials and Methods

2.1 Materials

Acetylcholine	Sigma, UK
Acetylsalicylic acid (aspirin)	Sigma, UK
Adenosine diphosphate	Labmedics, UK
Alexa647-conjugated secondary antibody	Invitrogen, UK
Anti-P-selectin-PE	eBioscience
Arachidonic acid	Sigma, UK
Ascorbic acid	Sigma, UK
Bovine serum albumin	Sigma, UK
Calcium Ionophone A23187	Sigma, UK
CHRONO-LUME	Labmedics, UK
CD61-APC	eBioscience, UK
CountBright Absolute counting beads	Invitrogen, UK
Epinephrine	Labmedica
Diclofenac	Sigma, UK
DEA/NONOate	Sigma, UK
Dimethyl Sulphoxide	VWR, UK
Epinephrine	Labmedics, UK
Ethanol	VWR, UK
FITC-conjugated anti-CD42b	eBioscience, UK
Formalin	Sigma, UK
Heparin	CP pharmaceuticals Ltd, UK
Horm Collagen	Takeda Austria
Lipopolysaccharide	Sigma, UK
Methanol-free formaldehyde	Fisher Scientific
PAC-1-FITC	BD Bioscience, UK

Prostaglandin I_2 / Prostacyclin	Tocris, UK
Phosphate buffered saline	Sigma, UK
Prasugrel active metabolite	Daitchi Sankyo, Japan
Ristocetin	Helena Bioscience, UK
Saline	Baxter, UK
Sodium Chloride	Sigma, UK
Sodium Nitroprusside	Sigma, UK
Tri-sodium citrate	Sigma, UK
Triton X-100	Sigma, UK
TRAP-6 amide	Bachem, UK
U46619	Enzo
VASP-P(Ser^{239}) primary antibody	Enzo Life-sciences, UK

2.2 Methods

Methods detailed below are applicable to several chapters of this thesis. Additional methods are described in the relevant chapter.

2.2.1 Ethics and consent

All experiments were approved by St Thomas's Hospital Research Committee (Ref. 07/Q0702/24) and all volunteers gave verbal and written consent before entering the studies.

2.2.2 Recruitment of study participants

All participants were screened for potential participation and after providing written consent a health status was carried out. Those volunteers deemed appropriate were recruited to participate in studies.

2.2.3 Blood collection

Blood was obtained by venepuncture performed in the ante-cubital fossa using a 19 gauge butterfly needle into a syringe containing 3.2% tri-sodium citrate, unless stated otherwise. Final ratios of anti-coagulant and blood were 1:9 to give a final concentration of 0.109 M.

2.2.4 Preparation of PRP and PPP

PRP was obtained by centrifugation of citrated whole blood at 175 × *g* for 15 minutes at 25°C. The PRP fraction was carefully removed as to not disturb the red blood cells or buffy coat. PPP was obtained by centrifugation of PRP at 1300 × *g* for 2 minutes.

2.2.5 Preparation of platelet agonists

ADP, TRAP-6 and U46619 were all prepared from 1 mM stock diluted in PBS. AA (100 mM stock in 100% ethanol) and epinephrine (1 mM) were diluted in 0.1% w/v ascorbic acid in PBS. Horm collagen (1 mg/ml) was diluted in isotonic glucose. Lyophilized ristocetin was reconstituted to 30 mg/ml in distilled H_2O. All agonists were prepared at concentrations 10 times the required final concentrations and added 1:10 to PRP.

2.2.6 Preparation of platelet inhibitors

DEA/NONOate (100 mM stock) and PGI_2 (10 mM stock) were diluted in NaOH (0.01 M) and also prepared at a concentration 10 times that required and added 1:10 to PRP.

2.2.7 Platelet aggregation

Platelet aggregation was measured by LTA, 96-well plate aggregation or optimul plate.

2.2.7.1 LTA

Aggregation responses were measured using a Bio/Data PAP-8E turbidimetric aggregometer (Alpha Laboratories, Eastleigh, UK). Prior to measuring aggregation response channels were blanked using a 250 µl sample of PPP (200 or 225 µl) plus a diluent (25 or 50 µl). Aggregation experiments were carried out at 37 °C with constant stirring at 1200 rpm. Cuvettes containing a stirrer bar were prepared with PRP (200 or 225 µl) and incubated for 2 minutes. 25 µl inhibitor solution was added to PRP (200 µl) samples testing inhibitory conditions and incubated for a further 1 minute. 25 µl agonist solution was then added (10:1 final concentration) to test maximal and final aggregation (MA and FA) responses measured over 5 minutes using 340 nm-light.

2.2.7.2 96-well plate aggregation

This modified light transmission method was used to assess platelet aggregation, as previously described [246]. 10 µl of agonist or vehicle were added to individual wells of a 96-well plate at 10 × final concentration. Four wells containing PRP and four previously prepared PPP wells served as controls corresponding to 100% and 0% aggregation, respectively. 100 µl samples of PRP were placed into individual wells containing agonists. The plate was then placed on a Tecan Sunrise (Tecan Trading AG, Switzerland) absorbance plate reader, set to measure absorbance at 595 nm in order to determine levels of platelet aggregation.

2.2.7.3 Optimul

Platelet aggregation was assessed using a previously reported methodological approach providing a standardised optical detection of platelet aggregation (optimul method) based upon 96-well plate aggregometry [247]. 4 wells containing PRP and 4 wells of PPP acted as control wells. 40 µl of PRP was added to each test well and the plate was then placed on a bioshaker at 1200 rpm at 37 ° C for 5 minutes. Platelet aggregation was determined immediately by changes in light absorbance detected at 595 nm by a Tecan Sunrise absorbance set (Tecan Trading AG, Switzerland).

2.2.8 ATP + ADP Release

Experiments were conducted in a lumi-aggregometer (560 CA, Chronolog, Havertown, PA, USA) to measure ADP + ATP secretion. Blank samples were prepared (200 µl PPP, 25 µl CHRONO LUME and 25 µl diluent) to set the baseline, acting as a reference range corresponding to 100% aggregation. Test samples (200 µl PRP, 25 µl CHRONO LUME and 25 µl diluent) containing stirrer bars were incubated for 2 minutes at 37 °C before being

stimulated with collagen (4 µg/ml) or TRAP-6 (25 µM). Chart v4.2 (AD Instruments, UK) was used to record aggregation and luminescence traces over 5 minutes at which point 4 nmol ATP was injected to act as a reference range for quantifying ATP release.

2.2.9 Flow cytometry

Flow cytometric analysis was used to quantify P-selectin and PAC-1 expression post-activation. CD61 was used to identify platelets. To make the antibody mix, 1 µl CD61 APC, 1.5 µl CD62p PE and 1.5 µl PAC-1-FITC were added to 46.5 µl saline per volunteer.

PRP samples were incubated and then stimulated with TRAP-6 (25 µM) in a 96-well plate. After gentle mixing at 37 °C, reactions were stopped through the addition of 10-fold excess of cold saline. Platelets were immediately stained with anti-CD61-allophycocyanin, PAC-1-FITC (BD Bioscience, Oxford, UK), and anti-P-selectin-PE (eBioscience) for 15 minutes at 4 °C and then fixed in 2% (v/v) formalin. PAC-1-FITC and anti-P-selectin-PE immunoreactivity were acquired on a FACSCalibur instrument (Becton Dickson, UK) using CellQuest software (Becton Dickinson, UK).

2.2.10 Statistical analyses

Data were analysed using Prism 6.0e (GraphPad Software, La Jolla, CA, USA). For tests of platelet aggregation, statistical significance was determined by two-way ANOVA with Dunnett's post-hoc test unless otherwise stated. Flow data were analysed using FlowJo v8.7 (Treestar, Ashland, USA) where the 'single platelet' population was gated based on forward scatter and CD61-APC immunoreactivity (FL-4 mean fluorescence intensity). Data sets are presented (Mean±SEM) and considered different if $p<0.05$. Additional statistical analyses are described in the relevant chapter.

Chapter 3: The *in vitro* synergy between $P2Y_{12}$ blockade, PGI_2 and NO

3.1 Introduction

It is well known that NO and PGI_2 independently amplify each other's effects and more recently it has been elucidated that the inhibitory actions of NO and PGI_2 are both independently potentiated by blockade of $P2Y_{12}$ receptors in platelets [237, 238]. In this first results chapter, I start to explore the hypothesis that the three-way synergy between PGI_2, NO and $P2Y_{12}$ receptor antagonism produces much more powerful platelet inhibition than $P2Y_{12}$ receptor blockade alone and hence, platelet function *in vivo* is a product of intrinsic platelet reactivity which can be modified by DAPT but also by the influence of the endothelial mediators, NO and PGI_2. Due to the mechanisms of action of these endothelial mediators, I also propose that there will be close associations of these anti-platelet agents to platelet cyclic nucleotide systems.

This relationship is firstly investigated using PRP from healthy volunteers treated with prasugrel active metabolite (PAM) *in vitro* to produce $P2Y_{12}$ blockade with and without aspirin. To these, NO or PGI_2 individually and in combination will be added to demonstrate their independent and combined effects on platelet inhibition using an array of tests of platelet function. Aggregation and ATP release experiments will be conducted and then flow cytometry to consider the downstream effects of $P2Y_{12}$ blockade, NO and PGI_2 by investigating levels of VASP phosphorylation, a downstream marker of PKG and PKA activation representative of cyclic nucleotide activity. I will then concentrate on the platelet cyclic nucleotide systems which are strongly effected by both PGI_2 and NO. Importantly, I will seek to define whether these interactions are synergistic in nature with the true potential of powerfully influencing platelet inhibition to a far greater extent than $P2Y_{12}$ blockade alone.

3.2 Methods

3.2.1 Recruitment of healthy volunteers

Potential blood donors aged 18-40, both male and female provided written consent and were subsequently screened. This process involved a detailed medical history, physical examination and observations including blood pressure, pulse rate and temperature. Those fulfilling the inclusion criteria with normal health profiles were recruited.

3.2.2 Preparation of platelet solutions

Blood was obtained by venepuncture and both PRP and PPP were prepared from whole anti-coagulated blood, as described in sections 2.2.3 and 2.2.4.

3.2.3 Treatment of PRP with anti-platelet drugs

Aspirin (ASA) was prepared in ethanol to a concentration of 100 mM and then diluted in PBS to a concentration of 3 mM. This was subsequently diluted in PRP 1:100 to obtain the required final concentration of 30 µM. PAM was prepared to a concentration of 10 mM in DMSO and diluted in PBS to a concentration of 600 µM. To achieve final concentrations of 1.5, 3 and 6 µM PAM solution this was further diluted 1:400, 1:200 and 1:100 in whole blood, respectively. PRP treated with PAM and/or ASA or vehicle was incubated at 37 °C for 30 minutes in a water bath before use in assays.

3.2.4 LTA

Platelet aggregation was determined by LTA, as described in section 2.2.7.1. Initially, PGI_2 (1-100 nM) or vehicle were added to vehicle or PAM (6 µM) treated PRP for 1 minute at 37 °C and stimulated with Horm collagen (30 µg/ml) or TRAP-6, specific for PAR-1 (30 µM). Aggregation experiments were repeated but with the addition of

DEA/NONOate (0.1-1000 nM) rather than PGI_2. These experiments were then repeated again but with the addition of either vehicle or both PGI_2 (1-8 nM) and DEA/NONOate (1-1000 nM) in PRP treated with either vehicle or PAM (6 μM) and stimulated with collagen (30 μg/ml) or TRAP-6 (30 μM). Following this, in PRP treated with vehicle or PAM (6 μM) with or without ASA (30 μM), aggregations to TRAP-6 (25 μM) and collagen (4 μg/ml) were measured after pre-incubation of PRP with vehicle (NaOH 0.01 M) DEA/NONOate (100 nM) or PGI_2 (1 nM) individually or the combination of DEA/NONOate and PGI_2 for 1 minute at 37 °C.

3.2.5 Isobolographic analysis

Inhibitory concentration curves for PGI_2 (1-8 nM) or DEA/NONOate (10 nM-1 μM) against aggregation induced by TRAP-6 (30 μM) or collagen (30 μg/mL), in the presence of vehicle or PAM (6 μM) were constructed with data fitted to a logistic equation using least-squares method. Derived data was used to generate isobolograms [320].

3.2.6 ADP + ATP Release

ADP + ATP release from platelets was assessed by lumi-aggregometry, as described in section 2.2.8. PRP treated with vehicle or PAM (6 μM) with or without ASA (30 μM) was pre-incubated with either vehicle, DEA/NONOate and/or PGI_2 as described above. ADP + ATP secretion was evaluated by luminescence in the presence of Chrono-Lume reagent after stimulation with TRAP-6 (25 μM) or Horm collagen (4 μg/ml).

3.2.7 VASP phosphorylation

PRP was pre-incubated with vehicle, ASA (30 μM), PAM (6 μM) or ASA+PAM and stimulated with collagen (4 μg/ml) or TRAP-6 (25 μM) in the presence of PGI_2 and/or

DEA/NONOate or vehicle. After 4 minutes the reaction was stopped with methanol-free formaldehyde (2% final). Platelets were permeabilized using 0.2% Triton X-100 and incubated with anti-VASP-P(Ser_{239}), Alexa647-conjugated secondary antibody and FITC-conjugated anti-CD42b, for 30 minutes each in turn. The platelet pellet was then resuspended in 0.9% saline. VASP-P (Ser_{239}) immunoreactivity was measured by flow cytometry, using a FACS-Calibur instrument.

3.2.8 cAMP and cGMP measurements

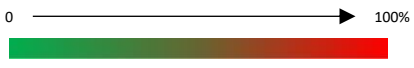
PAM (6 μM), ASA (30 μM), PAM+ASA or vehicle-treated PRP was stimulated with collagen (4 μg/mL) or TRAP-6 (25 μM) in the presence of PGI_2 and/or DEA/NONOate or vehicle. After 4 min, platelets were lysed with Triton-X-100 (0.625%) and treated with iso-butylmethylxanthine (IBMX; 500 μM) to inhibit phosphodiesterase activity. Potassium fluoride (0.5 M) was added to increase fluorophore stability in the homogenous time-resolved fluorescence (HTRF) based competitive immunoassays (Cisbio) used to determine cAMP and cGMP concentrations. HTRF combines fluorescence resonance energy transfer technology (FRET) with time-resolved measurement (TR). FRET is a technique based on the interactions between biomolecules assessed by coupling each with a fluorescent label and detecting the level of energy transfer. Proximity of two fluorophores is responsible for the specific signal emission. Elimination of background fluorescence is achieved by the addition of time-resolved measurement. In brief, as previously described by Degorce et al, the competitive cAMP assay combines anti-cAMP Ig labeled with a cryptate donor (anti-cAMP cyrptate conjugate) and cAMP labeled with acceptor d2 (cAMP d2 conjugate). Donor and acceptor are brought into proximity range by the binding of anti-cAMP Ig to cAMP. Energy is transferred from donor to acceptor on excitation of donor occurring at 337 nm

leading to donor and acceptor to generate emission at 620 nm and 665 nm, respectively. The level of cAMP generated is measured by competing with the cAMP d2 for antibody binding [321].

3.2.9 Heat map generation and scaling

The heat maps were created in Microsoft excel. Conditional formatting was applied to the cells containing the data range. A colour scale was chosen to allow visual representation of the underlying data. At one end of the scale, red represents 100% aggregation of platelets or maximal platelet ATP release. On the other end of the scale, green represents 0% platelet aggregation or no ATP release. There is a gradual change in the colour scale from red to dark red to brown to dark green through to green corresponding to decreasing percentage values. Dark brown represents the mid-way point (50%. The colour gradient was applied to the range of cells containing the data thus indicating where each data point falls within the data set. This allows a quick and powerful visual representation of my data alongside the other graphical resources and written text. The red (100%) and green (0%) are immediately differentiated and data points in between can be judged in conjunction with the underlying data and scale represented below.

The colour scale is as follows:



3.3 Results

3.3.1 Interactions between PGI_2 and $P2Y_{12}$ blockade with PAM on platelet aggregation

PRP pre-incubated with PAM (6 μM) or vehicle and treated with PGI_2 or vehicle was tested in the LTA to investigate the interactions between PGI_2 and $P2Y_{12}$ blockade. PGI_2 was added to PRP at concentrations of 1 nM, 10 nM or 100 nM before the PRP was stimulated with either ADP (20 μM), TRAP-6 (30 μM) or collagen (30 μg/ml) in the LTA (representative traces displayed in figure 3.1). In the presence of vehicle, full aggregation (>60%) was observed for all 3 agonists in PRP. For PRP stimulated with ADP (20 μM) the addition of PGI_2 (1 nM) reduced aggregation, which was abolished with higher concentrations of PGI_2. Pre-treatment of PRP with PAM (6 μM) however, led to near complete blockade of aggregation even in the presence of vehicle, while in the presence of all concentrations of PGI_2 aggregation was abolished. Aggregation induced by TRAP-6 (30 μM) in vehicle PRP was reduced by PGI_2 (10 nM), but not by PGI_2 at lower concentrations. In PAM treated PRP, however, aggregation was reduced by all 3 concentrations of PGI_2 (1 nM, 10 nM and 100 nM). In vehicle PRP, Collagen (30 μg/ml) caused strong aggregation in samples treated with vehicle or PGI_2 (1nM). The addition of PGI_2 (1 nM) to PAM (6 μM) treated PRP reduced aggregation, while under the same conditions both PGI_2 (10 and 100 nM) abolished aggregation. In vehicle treated PRP only the addition of PGI_2 (100 nM) fully inhibited platelet aggregation (Figure 3.2).

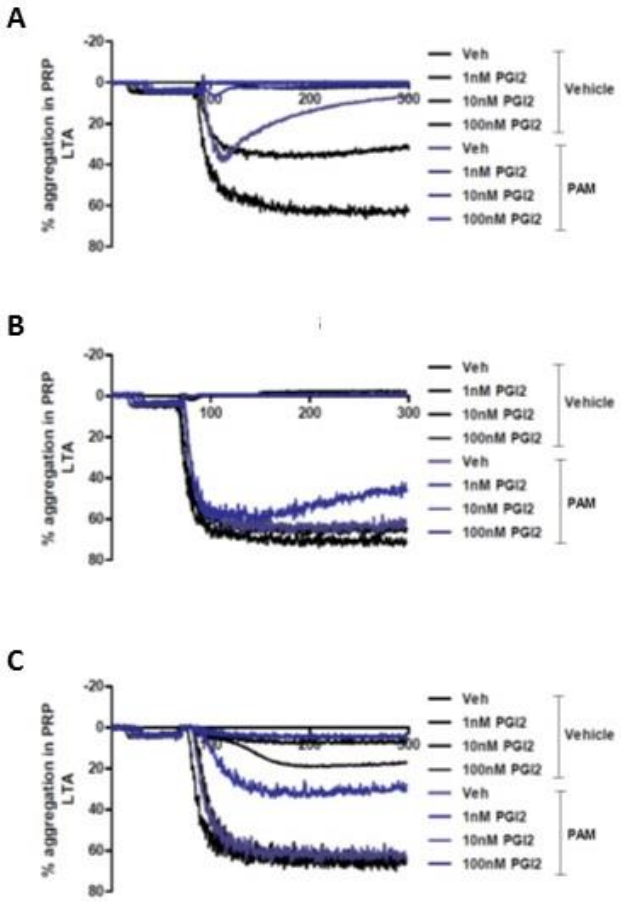


Figure 3.1: Influence of PAM and PGI_2 on platelet aggregation. LTA traces following stimulation with (A) ADP (20 µM), (B) TRAP-6 (30 µM) or (C) collagen (30 µg/ml) in PRP pre-incubated with PAM (6 µM) or vehicle with the addition of PGI_2 (1 nM, 10 nM or 100 nM) or vehicle.

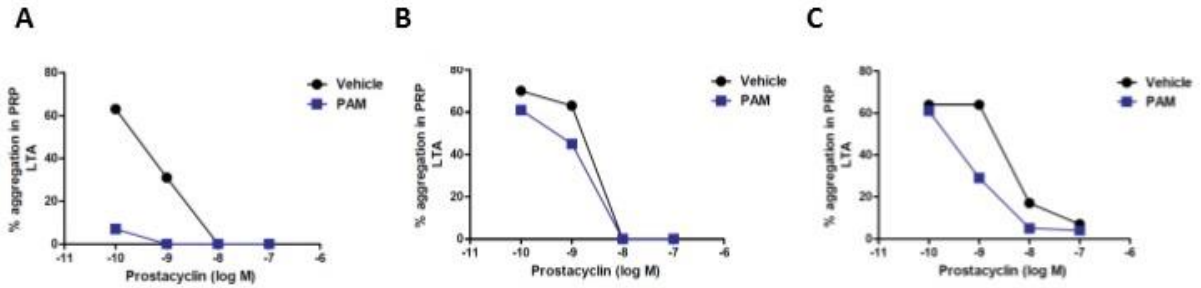


Figure 3.2: Influence of PAM and PGI_2 on platelet aggregation. Platelet aggregation in LTA following stimulation with (A) ADP (20 µM), (B) TRAP-6 (30 µg/ml) or (C) collagen (30 µM) in PRP pre-incubated with PAM (6 µM) or vehicle with the addition of PGI_2 (1 nM, 10 nM or 100 nM) or vehicle. N=1.

3.3.2 Interactions between NO and $P2Y_{12}$ blockade with PAM on platelet aggregation

PRP pre-incubated with PAM (6 μM) or vehicle and treated with DEA/NONOate, or vehicle was tested in the LTA to investigate the interactions between NO and $P2Y_{12}$ blockade. DEA/NONOate was added to PRP at concentrations of 0.1 nM, 10 nM and 1000 nM before the PRP was stimulated with either ADP (20 μM), TRAP-6 (30 μM) or collagen (30 μg/ml) in the LTA (representative traces displayed in figure 3.3). In the presence of vehicle, full aggregation (>60%) was observed for all 3 agonists in vehicle treated PRP. For PRP stimulated with ADP (20 μM) only the addition of DEA/NONOate (1000 nM) inhibited aggregation. Pre-treatment of PRP with PAM (6 μM) led to near complete blockade of aggregation in the presence of vehicle and all concentrations of DEA/NONOate. In vehicle treated PRP aggregation induced by TRAP-6 (30 μM) was only minimally reduced by DEA/NONOate (1000 nM). Pre-treatment with PAM (6 μM) however, led to near complete blockade of aggregation in the presence of DEA/NONOate (1000 nM). Collagen (30 μg/ml) caused strong aggregation in PRP treated with vehicle and all three concentrations of DEA/NONOate. The addition of DEA/NONOate (1000 nM) to PAM (6 μM) nearly fully inhibited platelet aggregation (Figure 3.4).

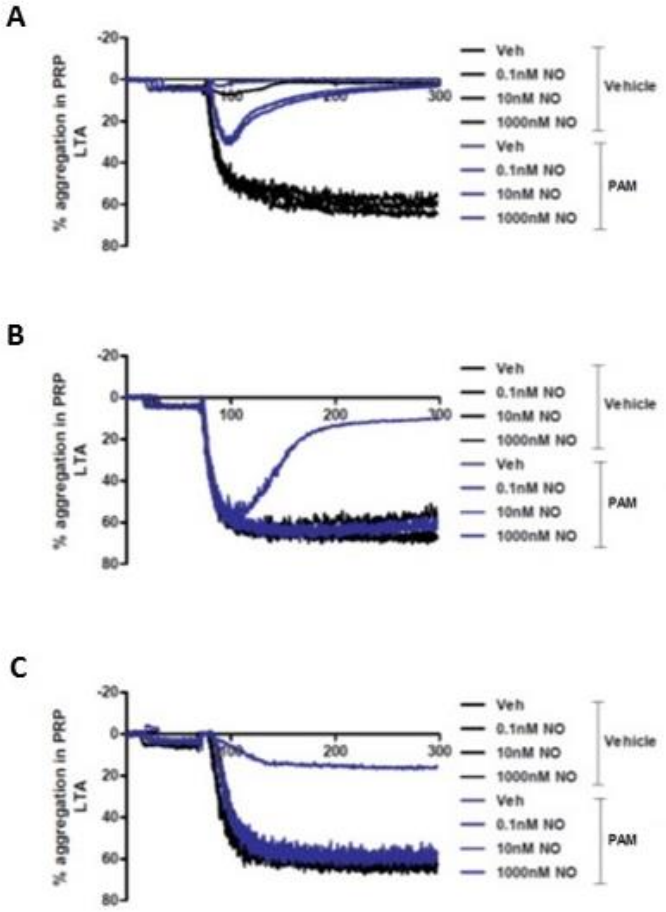


Figure 3.3: Influence of PAM and NO on platelet aggregation. LTA traces following stimulation with (A) ADP (20 µM), (B) TRAP-6 (30 µM) or (C) collagen (30 µg/ml) in PRP pre-incubated with PAM (6 µM) or vehicle with the addition of DEA/NONOate (0.1 nM, 10 nM or 1000 nM) or vehicle.

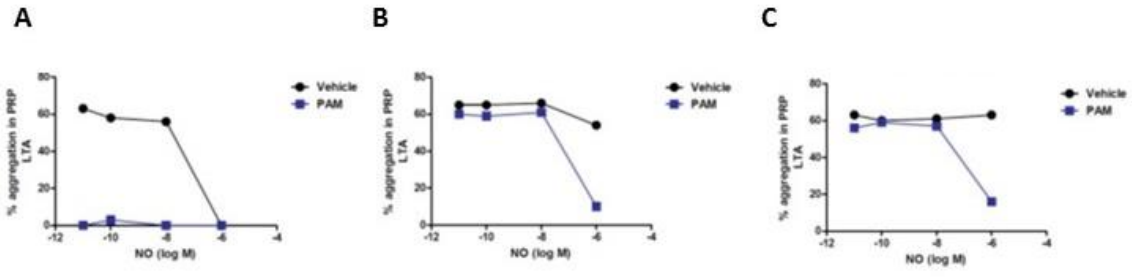


Figure 3.4: Influence of PAM and NO on platelet aggregation. Platelet aggregation in LTA following stimulation with (A) ADP (20 µM), (B) TRAP-6 (30 µM) or (C) collagen (30 µg/ml) in PRP pre-incubated with PAM (6 µM) or vehicle with the addition of DEA/NONOate (0.1 nM, 10 nM or 1000 nM) or vehicle. N=1.

3.3.3 Interactions between PGI_2, NO and $P2Y_{12}$ blockade with PAM on platelet aggregation

PRP pre-incubated with PAM (6 µM) or vehicle and treated with DEA/NONOate, PGI_2 or vehicle was tested in the LTA to investigate the interactions between PGI_2, NO and $P2Y_{12}$ blockade. DEA/NONOate was added to PRP at concentrations of 1-1000 nM to which PGI_2 (1-8 nM) or vehicle was added before the PRP was stimulated with either collagen (30 µg/ml) (Figure 3.5) or TRAP-6 (30 µM) (Figure 3.6). DEA/NONOate (1 nM) had little effect on collagen induced platelet aggregation. In vehicle PRP, DEA/NONOate (1000 nM) also had minimal effect however, in the presence of PGI_2 aggregation was reduced. PGI_2 (1-8 nM) increased platelet inhibition but pre-treatment with PAM led to much higher levels of platelet inhibition in all concentrations tested. In fact, aggregation was near abolished in PAM treated PRP in conditions of DEA/NONOate (10nM) and PGI_2 (2 nM) and above.

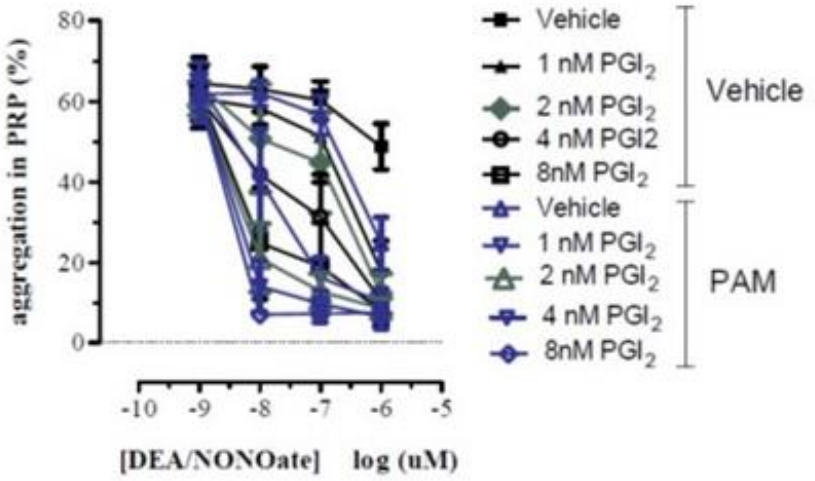


Figure 3.5: Influence of PAM, PGI_2 and NO on platelet aggregation. Platelet aggregation in LTA following stimulation with collagen (30 µg/ml). Platelets pre-incubated in PAM (6 µM) or vehicle and treated with PGI_2 (1 nM-8 nM) and DEA/NONOate (1 nM-1000 nM). Data are presented as final aggregation (%, mean±SEM). N=4

TRAP-6 induced full aggregation (>60%) in the presence of DEA/NONOate (1 nM) in both vehicle and PAM (6 µM) treated PRP. The addition of DEA/NONOate (1000 nM) to

vehicle PRP also led to high levels of aggregation which was reduced by PGI_2 (1-8 nM). In contrast, DEA/NONOate (1000 nM) led to markedly reduced platelet inhibition in PRP pre-incubated with PAM which was further reduced by PGI_2 (1-8 nM). Pre-treatment with PAM dramatically reduced platelet aggregation in all conditions tested. In vehicle PRP, aggregation was abolished by DEA/NONOate (1000 nM) and PGI_2 (8 nM). However DEA/NONOate (10 nM) and PGI_2 (4 nM) abolished aggregation in PAM (6 µM) treated PRP.

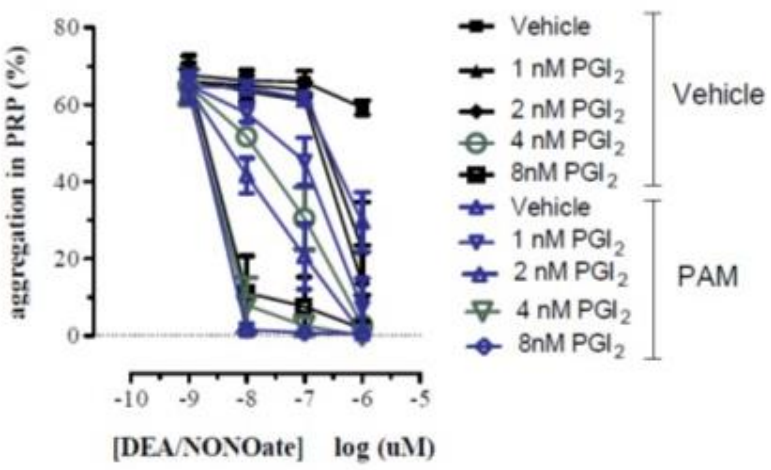


Figure 3.6: Influence of PAM, PGI_2 and NO on platelet aggregation. Platelet aggregation in LTA following stimulation with TRAP-6 (30 µM). Platelets pre-incubated in PAM (6 µM) or vehicle and treated with PGI_2 (1 nM-8 nM) and DEA/NONOate (1-1000 nM). Data are presented as final aggregation (%, mean, ±SEM). N=4.

3.3.4 Synergy between PGI_2, NO and $P2Y_{12}$ blockade

Isobolographic analyses indicated strong synergistic inhibition between DEA/NONOate and PGI_2 against platelet aggregation induced by both collagen (30 µg/mL; Figure 3.7A/B) and TRAP-6 amide (30 µM; Figure 3.7C/D), with isoboles curving strongly towards the axes. $P2Y_{12}$ blockade caused a further powerful (4-fold and 10-fold, respectively) enhancement in the synergy between DEA/NONOate and PGI_2 for the inhibition of aggregations induced by collagen (Figure 3.7B) and TRAP-6 (Figure 3.7D).

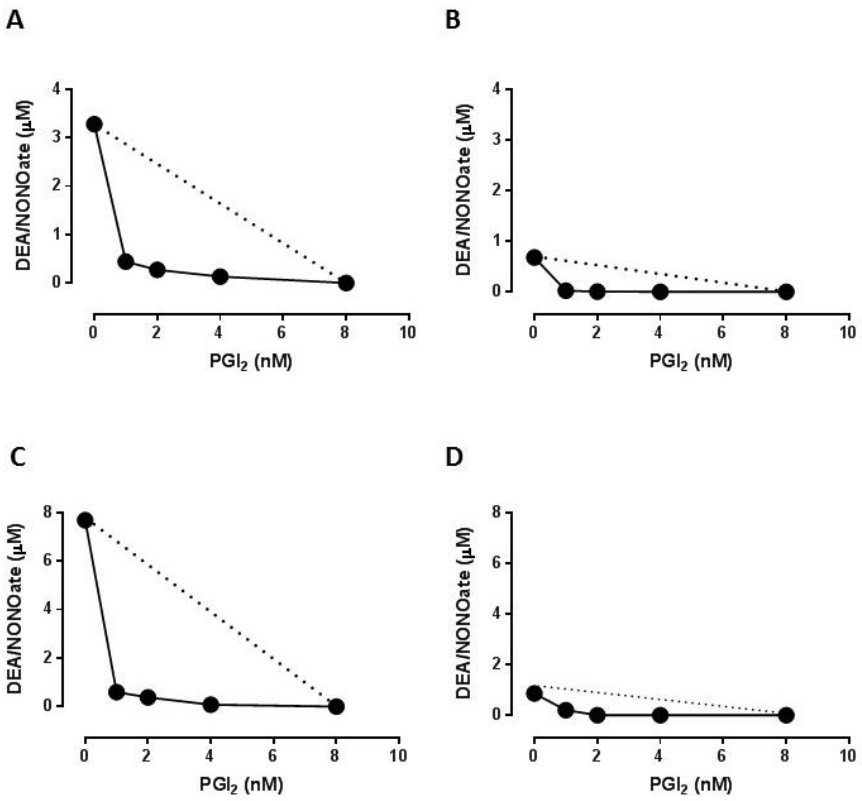


Figure 3.7: Synergism between NO, PGI_2 and PAM. IC_{50} isobolograms were generated by analyzing combinations of DEA/NONOate and PGI_2 required to produce a 50% inhibition of platelet aggregation stimulated by collagen (30 µg/ml) in the (A) absence and (B) presence of PAM (6 µM) and by TRAP-6 (30 µM) in the (C) absence and (D) presence of PAM (6 µM). The linear relationship is predicted by the arithmetic sum of the effect of either DEA/NONOate or PGI_2 alone, as described in the methods, and the experimental line curving towards the axes indicates a strong, synergistic relationship. n = 4 for each point.

3.3.5 Effects of PGI_2 and NO on platelet aggregation in the presence of submaximal $P2Y_{12}$ antagonism

PRP was taken from healthy volunteers and pre-incubated *in vitro* with PAM (1.5 µM, 3 µM and 6 µM) to represent 25%, 50% and 100% of the concentration of PAM required for total $P2Y_{12}$ receptor inhibition. Samples were treated with vehicle (NaOH, 0.01 M), DEA/NONOate (100 nM), PGI_2 (1 nM) or DEA/NONOate + PGI_2 and stimulated with ADP (20 µM), collagen (4 µg/ml) or TRAP-6 (25 µM). Aggregation in response to ADP (20 µM) was increasingly inhibited with increasing levels of PAM: control, 79±5%; PAM-25%, 70±5%; PAM-50%, 48±15%; PAM-100%, 4±1% ($p<0.05$).

Maximum platelet aggregation to collagen (4 μg/ml) in the presence of PAM-100% was reduced by the addition of DEA/NONOate (74±4% to 23±9%, $p<0.05$), PGI_2 (74±4% to 22±6%, $p<0.05$) and DEA/NONOate+PGI_2 (50±10% to 4±1%, $p<0.05$). Similarly, TRAP-6 (25 μM) induced aggregation was reduced in the presence of DEA/NONOate (70±2% to 36±6%, $p<0.05$), PGI_2 (67±2% to 35±3%, $p<0.05$) and DEA/NONOate+PGI_2 (63±3% to 4±2%, $p<0.05$). Indeed, even with submaximal, PAM-50% and PAM-25% $P2Y_{12}$ receptor inhibition, significant inhibition of platelet aggregation was found following addition of DEA/NONOate+PGI_2. Following collagen stimulation, DEA/NONOate+PGI_2 reduced aggregation (50±10% to 6±2%, $p<0.05$) and (50±10% to 21±10%, $p<0.05$) in the context of PAM-50% and PAM-25% receptor inhibition, respectively, compared to PAM-100% with vehicle (69±2% to 59±3%). DEA/NONOate+PGI_2 also reduced TRAP-6 induced aggregation in both PAM-25% (63±3% to 39±10%, $0<0.05$) and PAM-50% (63±3% to 20±6%, $p<0.05$) receptor inhibition compared to PAM-100% plus vehicle (69±2% to 59±3%). These results are displayed in graph form in figure 3.8A-F and are also expressed as heatmaps in figure 3.10A to aid visualisation of results (red represents maximum aggregation and green minimum aggregation).

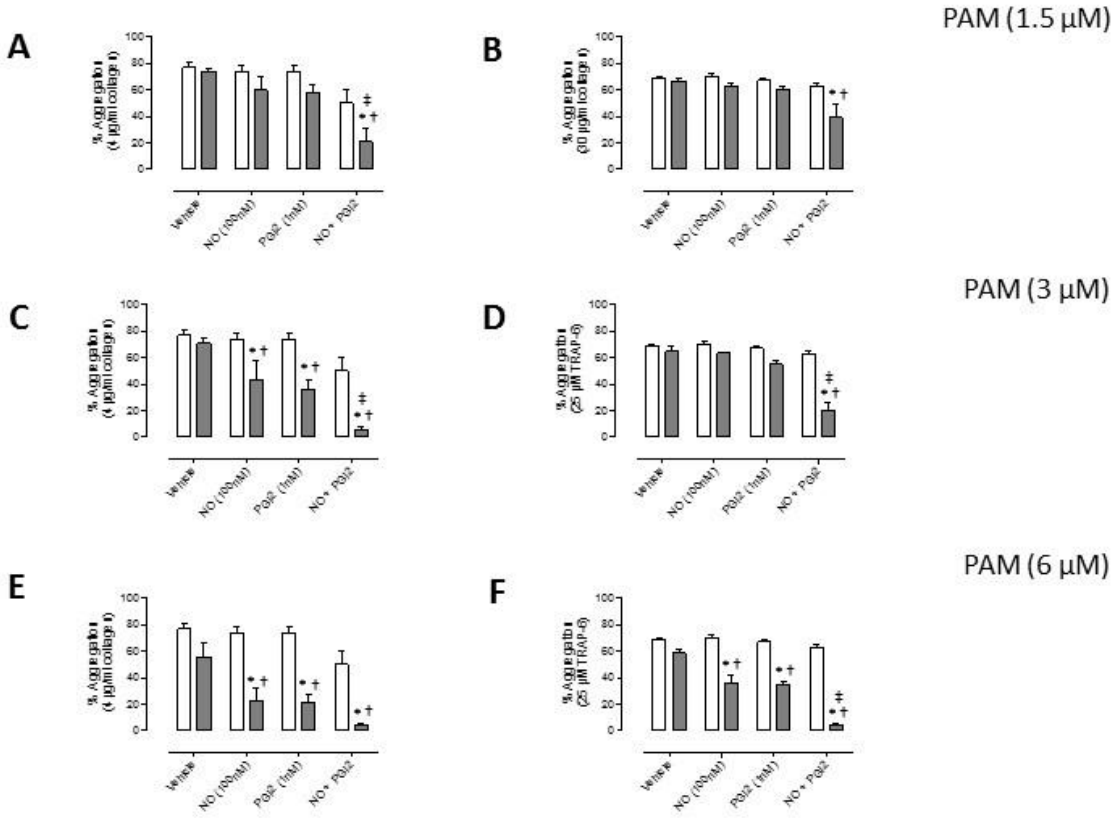


Figure 3.8: The *in vitro* effects of PAM on platelet aggregation. PRP from healthy volunteers was treated with PAM (1.5 µM, 3 µM and 6 µM) to represent 25%, 50% and 100% maximum concentration for total $P2Y_{12}$ receptor inhibition, respectively. Bar charts showing % final aggregation in response to (A/C/E) collagen (4 µg/mL) and (B/D/F) TRAP-6 (25 µM) in the presence of vehicle (NaOH, 0.01 M), NO (100 nM), PGI_2 (1 nM) or NO + PGI_2. Data are presented as final aggregation (%, mean, ±SEM). Significance is shown as * $p<0.05$ for pre- versus post-PAM. Significance is shown † $p<0.05$ vehicle versus inhibitor treatment. Significance is shown ‡ $p<0.05$ versus PAM+PGI_2-treated. N=4.

3.3.6 Effects of PGI_2 and NO on platelet aggregation in the presence of submaximal $P2Y_{12}$ antagonism and aspirin

PRP from healthy volunteers was pre-treated *in vitro* with PAM (1.5 µM, 3 µM and 6 µM) to represent 25%, 50% and 100% of the concentration of PAM required for total $P2Y_{12}$ receptor inhibition in the presence of aspirin (ASA). Samples were treated with vehicle (NaOH, 0.01 M), DEA/NONOate (100 nM), PGI_2 (1 nM) or DEA/NONOate + PGI_2 and stimulated with ADP (20 µM), collagen (4 µg/ml) or TRAP-6 (25 µM). Aggregation in response to ADP (20 µM) was increasingly inhibited with increasing levels of PAM+ASA:

control, 73±5%; ASA, 51±7%; ASA+PAM-25%, 33±11%; ASA+PAM-50%, 23±8%; ASA+PAM-100%, 7±1% ($p<0.05$). Maximum platelet aggregation to collagen (4 µg/ml) in the presence of ASA+PAM-100% was reduced by the addition of DEA/NONOate (66±8% to 3±2%, $p<0.05$), PGI_2 (64±8% to 5±2%, $p<0.05$) and DEA/NONOate+PGI_2 (43±10% to 2±1%, $p<0.05$). Similarly, TRAP-6 (25 µM) induced aggregation was reduced in the presence of DEA/NONOate (72±4% to 31±12%, $p<0.05$), PGI_2 (71±4% to 46±7%, $p<0.05$) and DEA/NONOate+PGI_2 (67±5% to 15±7%, $p<0.05$). Again, in the context of submaximal, PAM-50% and PAM-25% $P2Y_{12}$ receptor inhibition, the addition of DEA/NONOate and PGI_2 led to significant inhibition of platelet aggregation. Although ASA alone plus DEA/NONOate+PGI_2 inhibited collagen-induced platelet aggregation, TRAP-6-induced aggregation was only significantly inhibited with the addition of DEA/NONOate+PGI_2 to ASA+PAM-100% (67±5 to 15±7%, $p<0.05$), ASA+PAM-50% (67±6% to 33±15%, $p<0.05$), and even ASA+PAM-25% (67±5% to 36±13%, $p<0.05$) as compared to ASA (67±5% to 50±13%) and vehicle with ASA+PAM-100% (75±4% to 56±3%). Again, to aid in visualisation of results these data are expressed as heatmaps in figure 3.10B and also below in graph form in figure 3.9A-H.

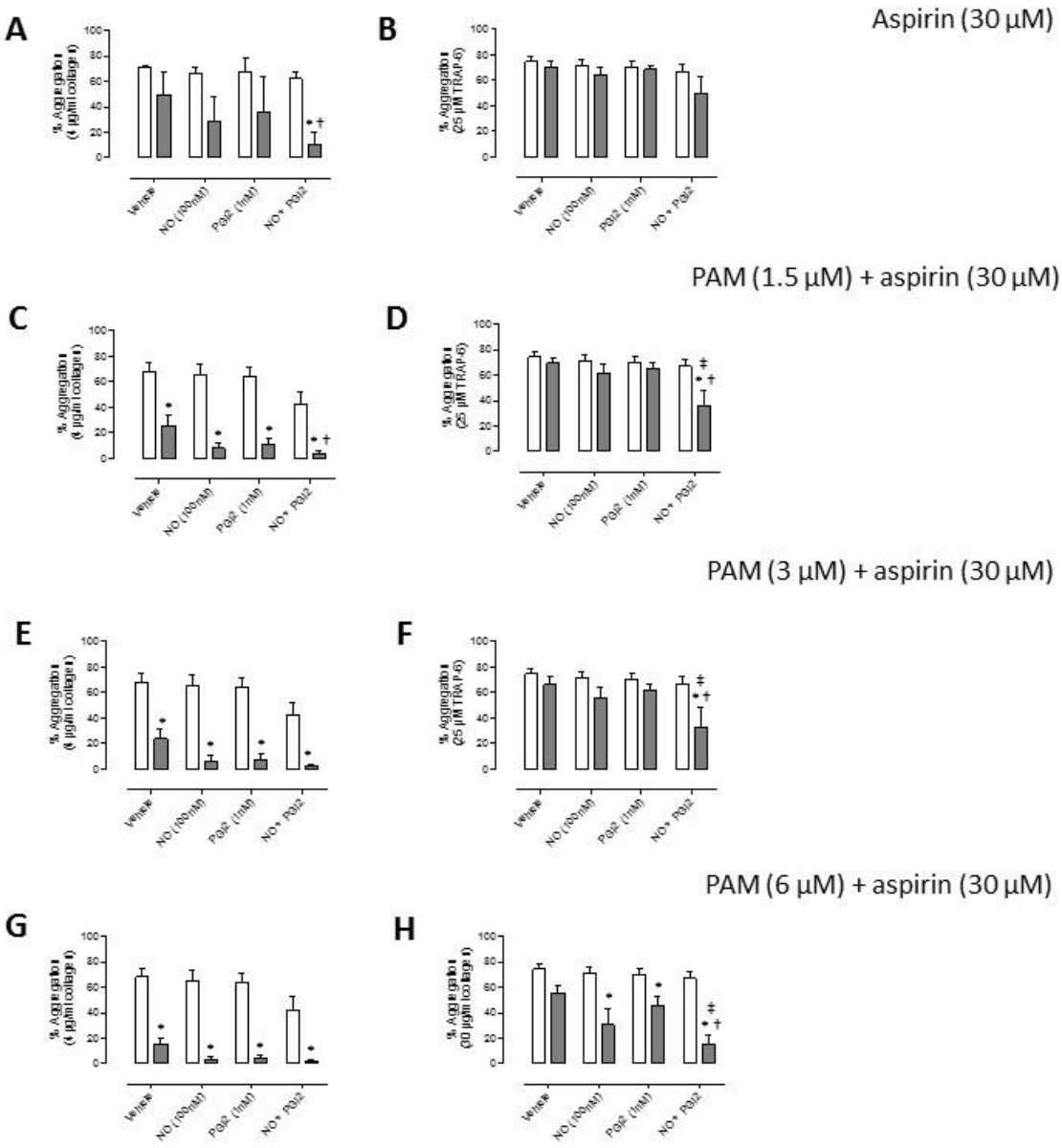


Figure 3.9: The *in vitro* effects of aspirin and PAM on platelet aggregation. PRP from healthy volunteers was treated with PAM (1.5 µM, 3 µM and 6 µM) to represent 25%, 50% and 100% maximum concentration for total $P2Y_{12}$ receptor inhibition, respectively. Bar charts showing % final aggregation in response to (A/C/E/G) collagen (4 µg/mL) and (B/D/F/H) TRAP-6 (25 µM) in the presence of vehicle (NaOH, 0.01 M), NO (100 nM), PGI_2 (1 nM) or NO + PGI_2 in the presence of aspirin (30 µM). Data are presented as final aggregation (%, mean, ±SEM). Significance is shown as * $p<0.05$ for pre- versus post-PAM. Significance is shown † $p<0.05$ vehicle versus inhibitor treatment. Significance is shown ‡ $p<0.05$ versus PAM+PGI_2-treated. N=4.

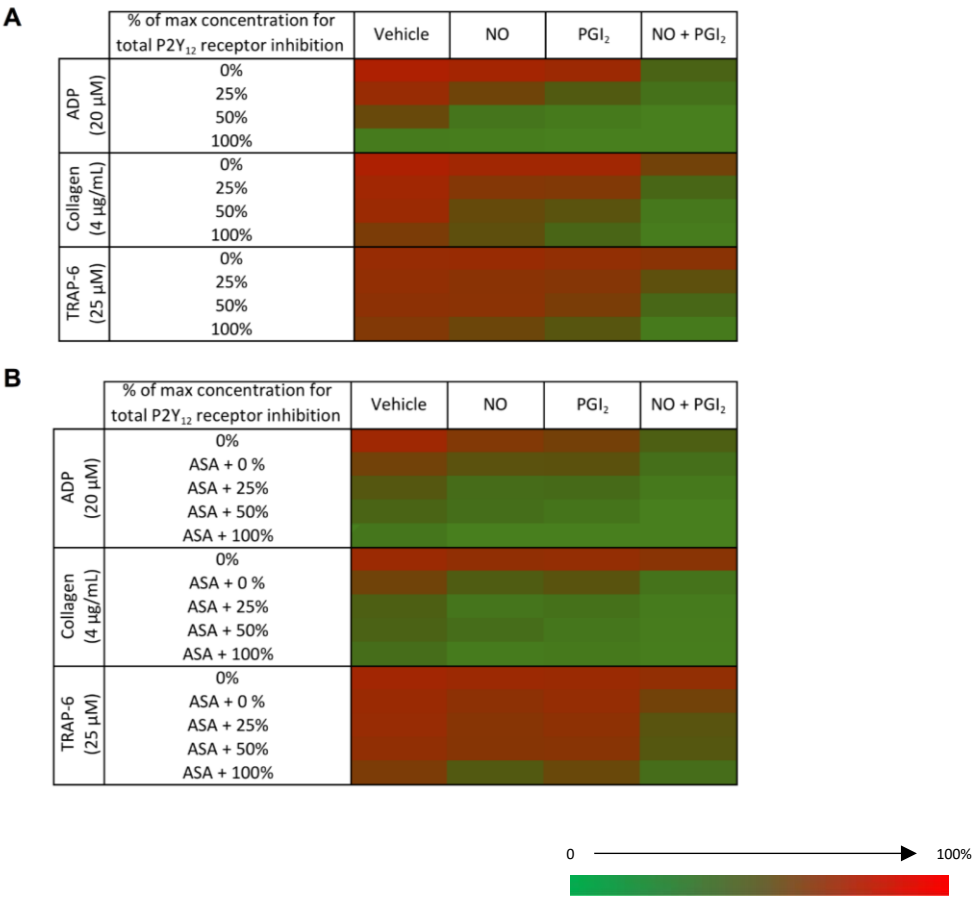


Figure 3.10: The *in vitro* effects of PAM with and without aspirin on platelet aggregation. PRP from healthy volunteers (n = 4) was treated with PAM (1.5 µM, 3 µM and 6 µM) to represent 25%, 50% and 100% maximum concentration for total $P2Y_{12}$ receptor inhibition, respectively. Heatmaps showing % final aggregation in response to ADP (20 µM), collagen (4 µg/mL) and TRAP-6 (25 µM) in the presence of vehicle (NaOH, 0.01 M), NO (100 nM), PGI_2 (1 nM) or NO + PGI_2 were generated in the (A) absence and (B) presence of aspirin (30 µM). Red represents maximum aggregation and green shows minimum aggregation with each cell representing data from 4 subjects.

3.3.7 Effects of PGI_2 and NO on ATP release in the presence of submaximal $P2Y_{12}$ antagonism

Collagen (4 µg/mL) induced ATP release was significantly inhibited by DEA/NONOate+PGI_2 in the presence of full and partial $P2Y_{12}$ receptor inhibition; PAM-100% (6.0±1.3 nM to 2.3±0.5 nM, $p<0.05$), PAM-50% (6.0±1.3 nM to 4.0±1.8 nM, $p<0.05$) and PAM-25% (6.0±1.3 nM to 4.1±1.7 nM, $p<0.05$), but not by DEA/NONOate or PGI_2 alone. These results compare to (10.8±3.3 to 8.2±2.0) for PAM-100% receptor inhibition

with vehicle. DEA/NONOate+PGI_2 also reduced ATP release following TRAP-6 (25 µM) stimulation in the context of full $P2Y_{12}$ receptor inhibition PAM-100% (9.7±1.5 nM to 3.0±1.5 nM, $p<0.05$). Althou+PGI_2 did not significantly reduce ATP release in the context of partial $P2Y_{12}$ receptor blockade, PAM-50% (6.0±1.3 nM to 4.0±1.8 nM) and PAM-25% (6.0±1.3 nM to 4.11.7), ATP release was lower in these sub-optimal $P2Y_{12}$ blockade conditions as compared to PAM-100% plus vehicle (13.0±1.0 nM to 11.7±3.7 nM). Figure 3.11A-F displays these results in bar graph format and figure 3.13A as heatmaps.

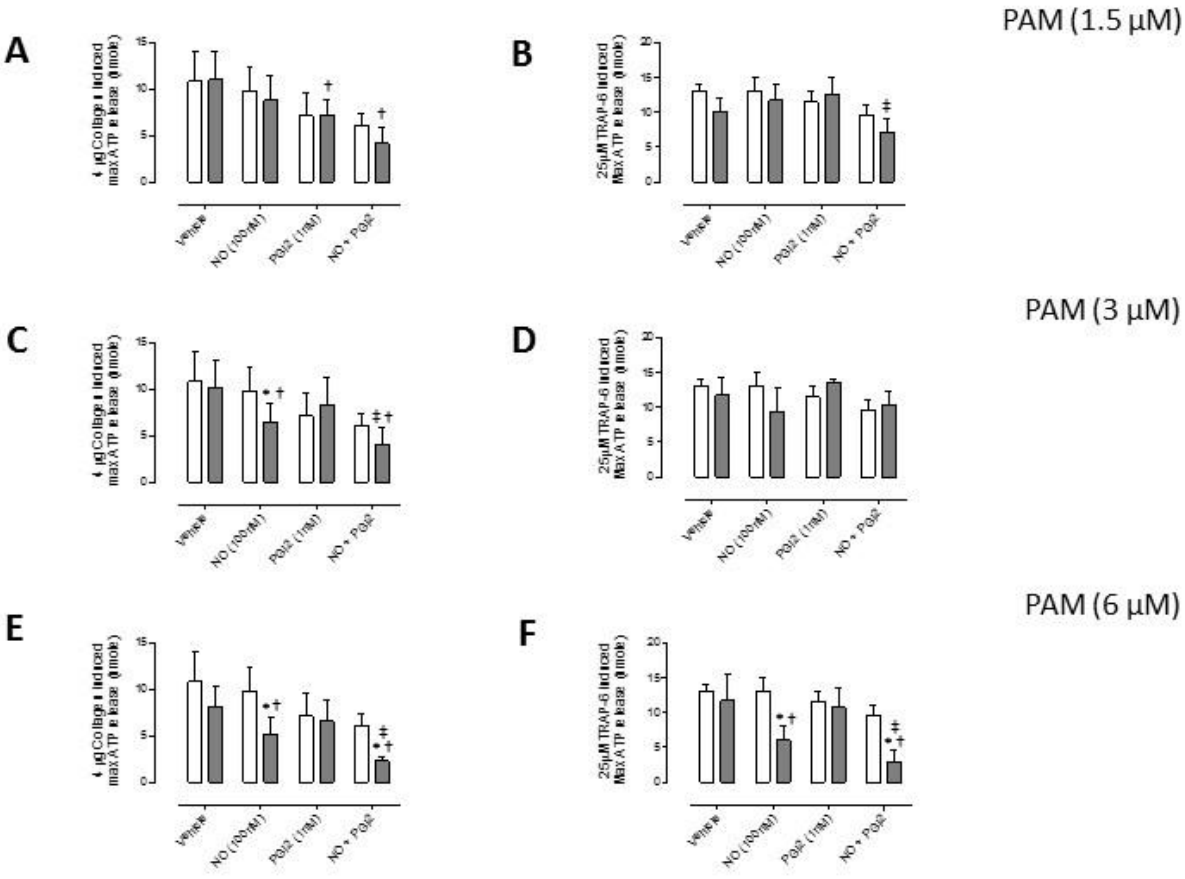


Figure 3.11: The *in vitro* effects of PAM on ATP release. PRP from healthy volunteers was treated with PAM (1.5 µM, 3 µM and 6 µM) to represent 25%, 50% and 100% maximum concentration for total $P2Y_{12}$ receptor inhibition, respectively. Bar graphs showing ATP release in response to (A/C/E) collagen (4 µg/mL) and (B/D/F) TRAP-6 (25 µM) in the presence of vehicle (NaOH, 0.01 M), NO (10 0 nM), PGI_2 (1 nM) or NO + PGI_2. Data are presented as ATP release (nmole, mean, ±SEM). Significance is shown as * $p<0.05$ for

pre- versus post-PAM. Significance is shown † p<0.05 vehicle versus inhibitor treatment. Significance is shown ‡ p<0.05 versus PAM+PGI_2-treated.N=4.

3.3.8 Effects of PGI_2 and NO on ATP release in the presence of submaximal $P2Y_{12}$ antagonism and aspirin

Similarly to the above results for PAM alone, DEA/NONOate+PGI_2 reduced collagen induced ATP release in the presence of submaximal levels of $P2Y_{12}$ blockade (ASA+PAM-0%, 3.3±0.2 nM; ASA+PAM-25%, 1.9±0.3 nM, p<0.05; ASA+PAM-50%, 2.1±0.3, p<0.05; ASA+PAM-100%, 1.8±0.2 nM, p<0.05). Following TRAP-6 (25 μM) stimulation, even in the presence of full $P2Y_{12}$ inhibition, neither DEA/NONOate nor PGI_2 alone reduced ATP release. However, in combination ATP release was significantly reduced in the presence of full $P2Y_{12}$ blockade (ASA+PAM-100%, 4.9±1.1 nM, p<0.05) and in the presence of partial $P2Y_{12}$ blockade (ASA+PAM-50%, 5.3±1.2 nM, p<0.05; ASA+PAM-25%, 6.7±1.1 nM, p<0.05). Notably, aspirin therapy alone did not produce a significant reduction in ATP release (ASA+PAM-0%, 7.7±1.2 nM). Figure 3.12A-H display these results as bar graphs and figure 3.13B.

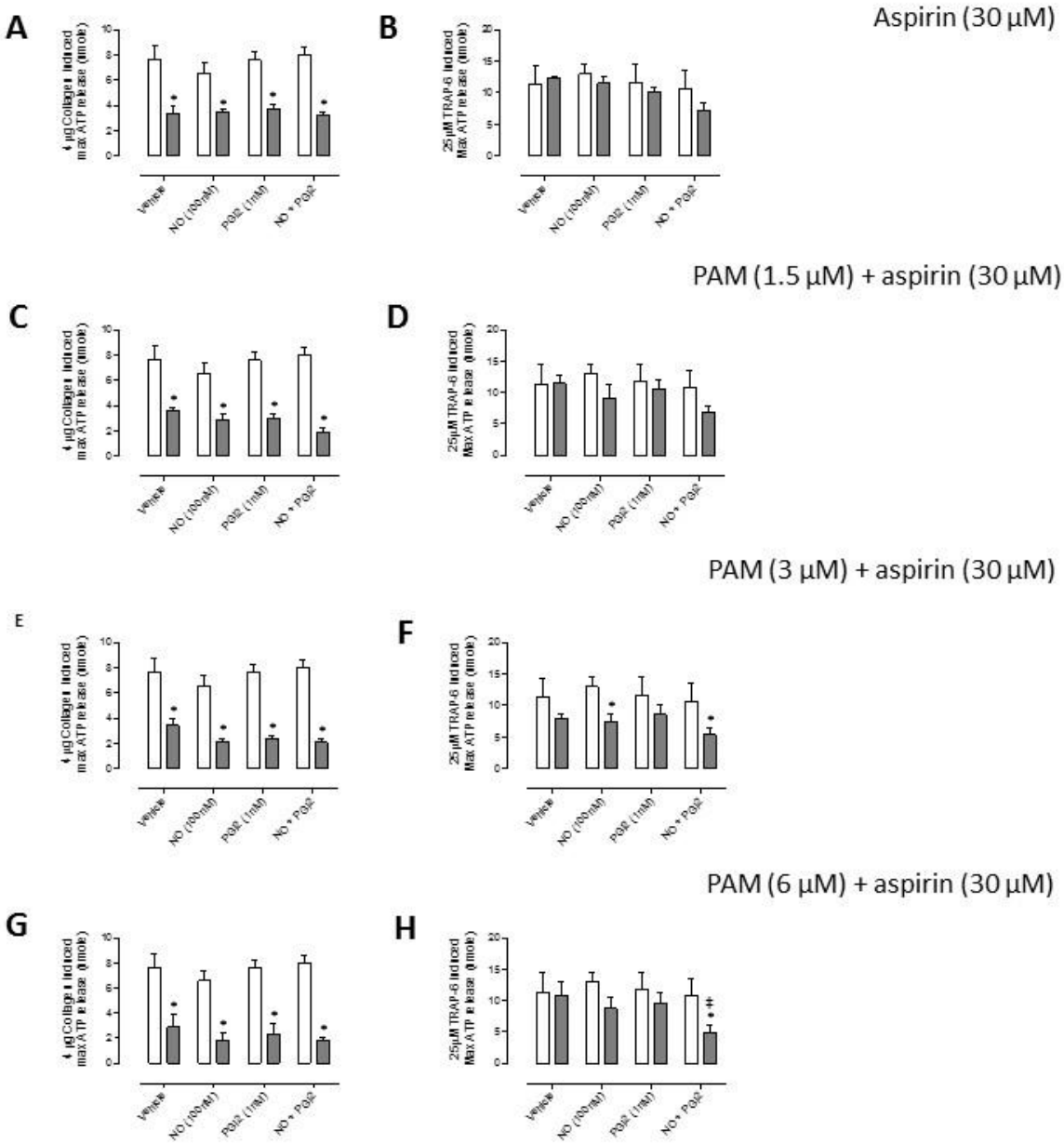


Figure 3.12: The *in vitro* effects of aspirin and PAM on ATP release. PRP from healthy volunteers was treated with PAM (1.5 µM, 3 µM and 6 µM) to represent 25%, 50% and 100% maximum concentration for total $P2Y_{12}$ receptor inhibition, respectively. Bargraphs showing ATP release in response to (A/C/E/G) collagen (4 µg/mL) and (B/D/F/H) TRAP-6 (25 µM) in the presence of vehicle (NaOH, 0.01 M), NO (100 nM), PGI_2 (1 nM) or NO + PGI_2 in the presence of aspirin (30 µM). Data are presented as ATP release (nmole, mean, ±SEM). Significance is shown as * $p<0.05$ for pre- versus post-PAM. Significance is shown † $p<0.05$ vehicle versus inhibitor treatment. Significance is shown ‡ $p<0.05$ versus PAM+PGI_2-treated.N=4.

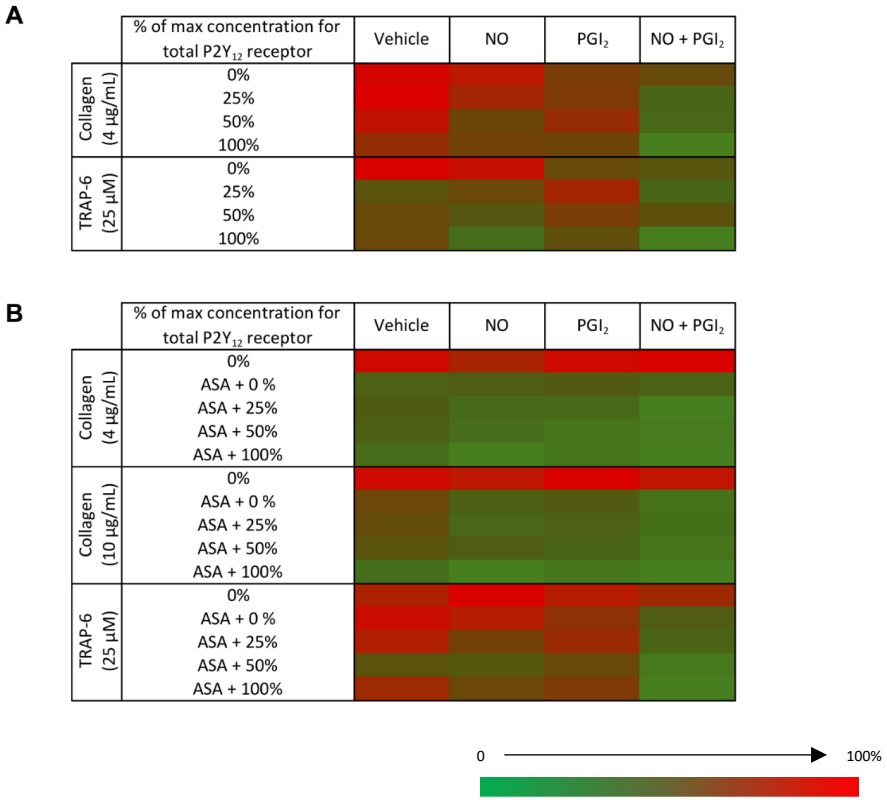


Figure 3.13: The *in vitro* effects of PAM with and without aspirin on ATP release. PRP from healthy volunteers (n = 4) was treated with PAM (1.5 µM, 3 µM and 6 µM) to represent 25%, 50% and 100% maximum concentration for total $P2Y_{12}$ receptor inhibition, respectively. Heatmaps showing ATP release in response to collagen (4 and 10 µg/mL) and TRAP-6 (25 µM) in the presence of vehicle (NaOH, 0.01 M), NO (100 nM), PGI_2 (1 nM) or NO + PGI_2 were generated in the (A) absence and (B) presence of aspirin (30 µM). Red represents maximum ATP release and green shows minimum ATP release with each cell representing data from 4 subjects.

3.3.9 Effects of $P2Y_{12}$ blockade, PGI_2 and NO on VASP-phosphorylation

To further investigate the effects of $P2Y_{12}$ blockade on the downstream pathways of PGI_2 and NO the levels of phospho (Ser^{239})-VASP, a downstream marker of PKG and PKA activation, were measured. Figure 3.14 illustrates representative control data for TRAP-6 (25 µM) induced VASP phosphorylation experiments. This was done in PRP incubated with PAM (6 µM) and/or ASA (30 µM) or vehicle, treated with vehicle NaOH (0.01 M), DEA/NONOate (100 nM), PGI_2 (1 nM), or DEA/NONOate + PGI_2 and stimulated with collagen (4 µg/ml) or TRAP-6 (25 µM) (Figure 3.15).

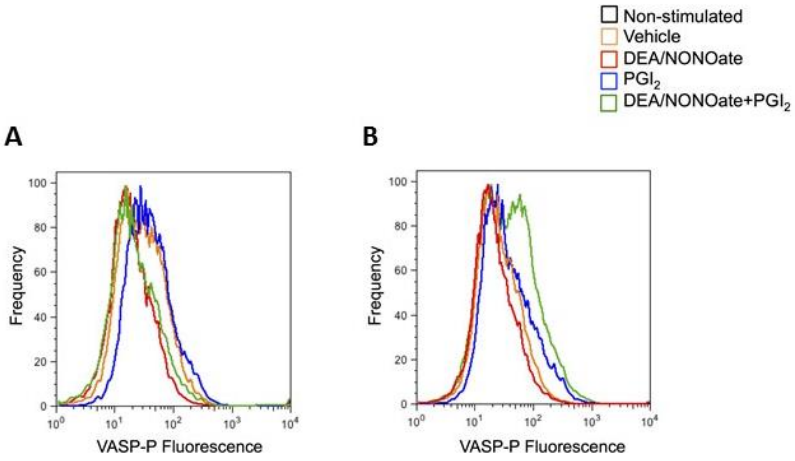


Figure 3.14: Representative control data for VASP phosphorylation experiments. VASP phosphorylation (Ser239) in the (A) absence and (B) presence of DAPT was measured in TRAP-6 (25 µM) stimulated PRP treated with vehicle NaOH (0.01 M), DEA/NONOate (100 nM), PGI_2 (1 nM), or DEA/NONOate + PGI_2 by flow cytometry. Histograms are representative of n = 4.

Following collagen stimulation, VASP phosphorylation remained unchanged and no significant changes were found in any of the conditions studied (Figure 3.15A). Following TRAP-6 stimulation, however, in all the treatment conditions tested in the presence of PAM, VASP-phosphorylation was increased. This increase was notable in the presence of PGI_2, 28±5 to 47±15 units ($p<0.05$). However, the largest significant increase in VASP-phosphorylation was detected in the DEA/NONOate+PGI_2 treated group (27±4 to 46±11 units, $p<0.05$). DEA/NONOate+PGI_2 also significantly affected VASP levels in platelets pre-treated with DAPT (27±4 to 34±4, $p<0.05$) (Figure 3.15B).

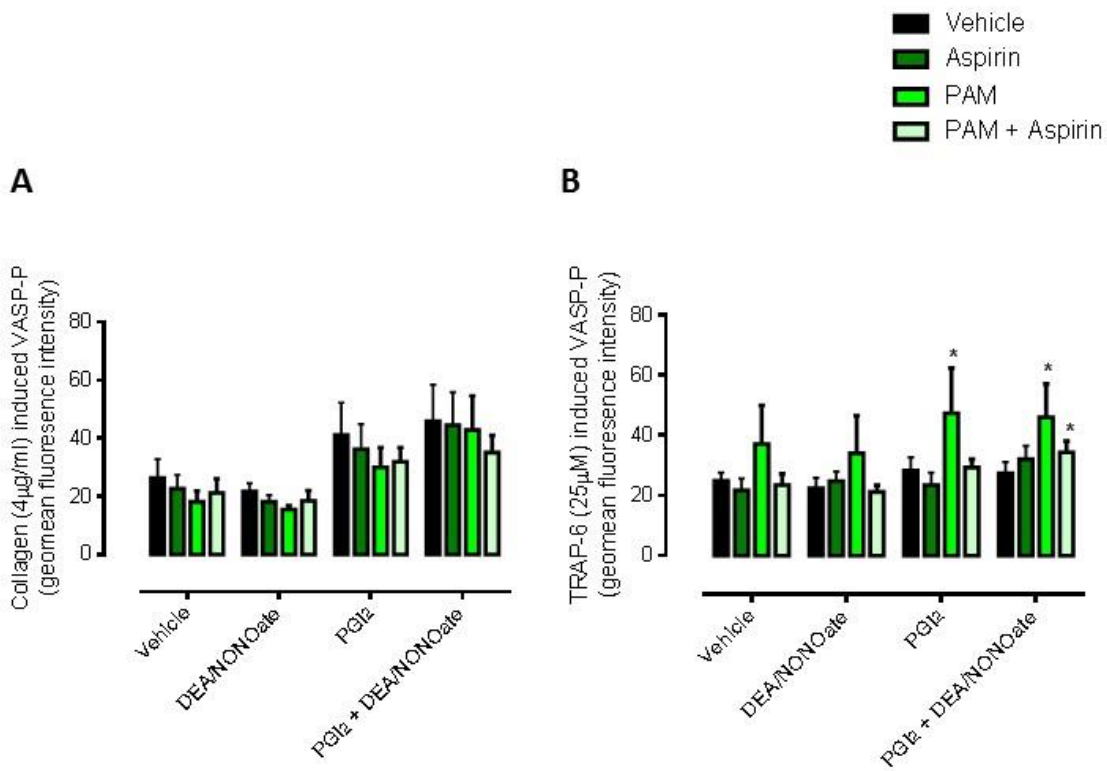


Figure 3.15: The *in vitro* effects of aspirin and PAM on VASP phosphorylation. PRP from healthy volunteers (n = 4) was treated with aspirin (30 µM), PAM (6 µM), both or vehicle followed by addition of vehicle (NaOH, 0.01 M), DEA/NONOate (100 nM), PGI_2 (1 nM) or DEA/NONOate + PGI_2. Following stimulation by (A) collagen (4 µg/ml) or (B) TRAP-6 (25 µM) phospho (Ser239) VASP levels were then determined by flow cytometry as a composite of cGMP and cAMP responses. Significance is shown as * $p < 0.05$ vs. non-treated.

3.3.10 Involvement of cAMP and cGMP in the synergistic effects of $P2Y_{12}$ blockade, PGI_2 and NO

No significant changes were found in cGMP levels in platelets in response to DEA/NONOate and/or PGI_2 after incubation with ASA, PAM or ASA+PAM during platelet aggregation stimulated by collagen (4 µg/ml; Figure 3.16C) or TRAP-6 (25 µM; Figure 3.16D). In collagen-stimulated platelets, basal cAMP levels (0.8±0.1 nM) were not altered by DEA/NONOate, but were significantly increased by PGI_2 (2.6±0.3 nM, $p<0.05$) and even more so by the combination of DEA/NONOate+PGI_2 (4.9±0.6 nM, $p<0.05$). However, neither PAM, ASA nor PAM+ASA altered the cAMP responses of collagen stimulated platelets (Figure 3.16A). In contrast, in TRAP-6-stimulated platelets,

DEA/NONOate, PGI_2 and their combination did not elevate cAMP levels in vehicle or ASA groups, but PGI_2 did in the presence of PAM (1.0±0.1 nM to 2.3±0.2 nM, $p<0.05$) and PAM+ASA (1.4±0.5 nM to 2.2±0.4 nM, $p<0.05$). This response was further enhanced by addition of PGI_2+DEA/NONOate (PAM, 3.4±0.6 nM; PAM+ASA, 3.5±0.6 nM; $p<0.05$; Figure 3.16B).

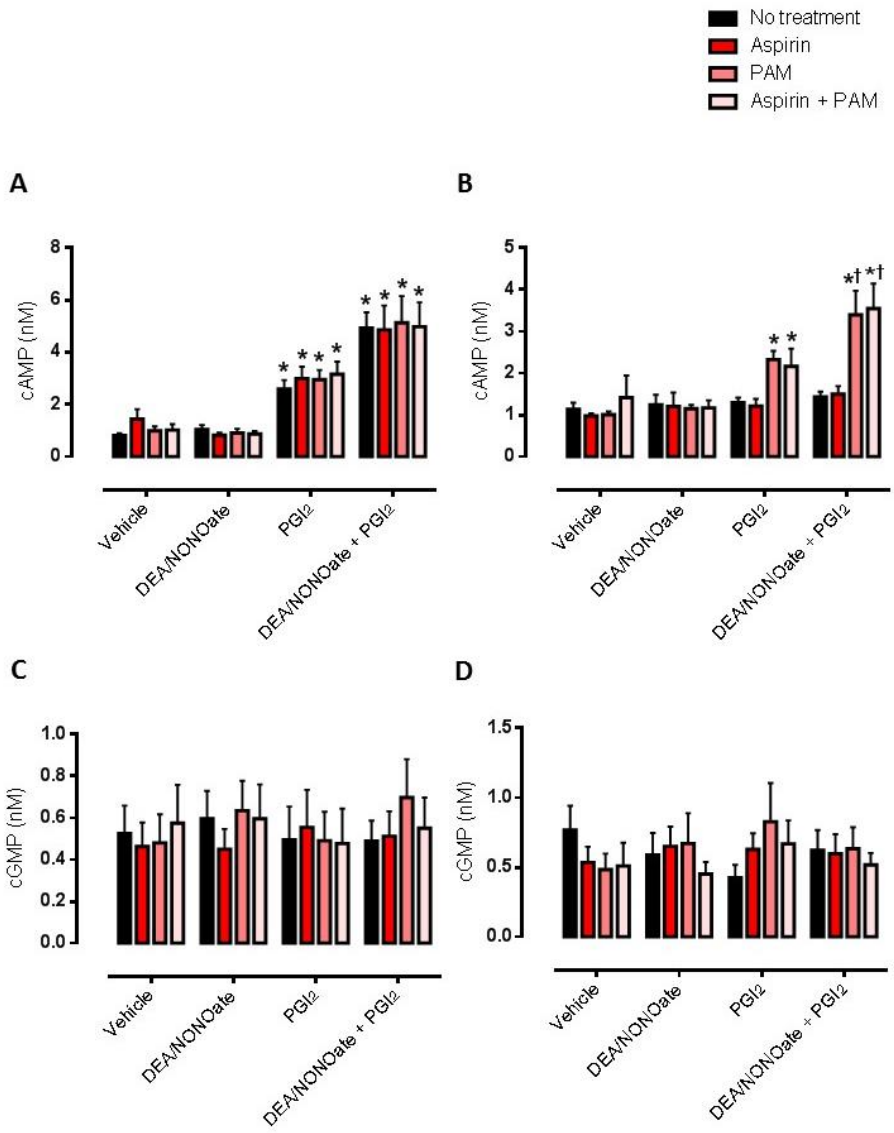


Figure 3.16: The *in vitro* effects of aspirin and PAM on cyclic nucleotide levels. PRP from healthy volunteers (n = 4) was treated with aspirin (30 µM), PAM (6 µM), both or vehicle followed by addition of vehicle (NaOH, 0.01 M), NO (100 nM), PGI_2 (1 nM) or NO + PGI_2. cAMP levels were then measured following stimulation by (A) collagen (4 µg ml) and (B) TRAP-6 (25 µM) as were cGMP levels following (C) collagen (4 µg ml) and (D) TRAP-6 (25 µM). Significance is shown as * $P < 0.05$ vs. non-treated and † $p < 0.05$ PGI_2 with corresponding PAM or aspirin + PAM.

3.4 Discussion

It is well known that NO and PGI_2 synergise to inhibit platelets [74] and it has been demonstrated that $P2Y_{12}$ antagonists potentiate the inhibitory actions of both PGI_2, dependent upon cAMP [237], and NO, dependent upon cGMP generation [238]. I therefore, reasoned that differences in the levels of NO and PGI_2 in the presence of the same levels of $P2Y_{12}$ receptor blockade will produce different levels of platelet inhibition in an individual patient. Furthermore, that the strong synergies between $P2Y_{12}$ inhibitors and the cAMP and cGMP signaling systems mean that the *in vivo* platelet reactivity in patients receiving DAPT will be a function of the level of $P2Y_{12}$ receptor blockade and the levels of endothelial-derived NO and PGI_2.

Here, I have started to investigate this hypothesis *in vitro* using PAM with and without aspirin to show that strong interactions between $P2Y_{12}$ receptor blockade and endothelial derived mediators produce profound inhibitory effects upon platelets, far more than $P2Y_{12}$ blockade acting alone. These studies highlight that the interactions of NO, PGI_2 and $P2Y_{12}$ receptor blockade in inhibiting platelets are markedly synergistic as noted by isobolographic analysis and measures of aggregation, ATP release, VASP phosphorylation and cyclic nucleotide expression.

Firstly, the results show that blockade of platelet $P2Y_{12}$ receptors markedly enhances the antiplatelet potency of both PGI_2 and NO individually, causing a 10- to 1000-fold increase in inhibitory activity against platelet aggregation in response to activation of receptors for either thrombin, collagen or ADP, and that this is even further enhanced when PGI_2 and NO are applied in combination. This was demonstrated over a range of

concentrations of NO (0.01-1000 nM) and PGI_2 (1 nM- 100 nM). High concentrations of thrombin (30 μM), collagen (30 μM) and ADP (20 μM) were used in order to display the true potency of these powerful interactions in the context of full $P2Y_{12}$ blockade which is achieved using PAM (6 μM).

To establish whether the interactions observed between NO, PGI_2 and $P2Y_{12}$ blockade for inhibition of thrombin and collagen induced platelet aggregation were additive or synergistic, isobolographic analyses were conducted [320]. To do this, matched aggregation studies were performed by LTA, using high concentrations of the platelet agonists collagen (30 μg/ml) and TRAP-6 (30 μM). Aggregation was measured in the absence and presence of full $P2Y_{12}$ receptor blockade using PAM (6 μM). From these data, isobolograms were constructed indicating the concentrations of DEA/NONOate and PGI_2 required, in combination, to produce 50% inhibition of platelet aggregation with and without PAM (6 μM). A simple additive relationship is indicated on an isobologram by a straight line, i.e. a relationship where a reduction in concentration of one inhibitor necessitates a linear increase in the concentration of the other to maintain the same level of inhibition. However, the isobolograms generated here displaying the interaction between DEA/NONOate and PGI_2 indicate a powerful synergy between these two inhibitors with the isoboles curving markedly toward the axes. Importantly, for both agonists tested the blockade of $P2Y_{12}$ receptors led to a further powerful enhancement of platelet inhibition. In fact, in the presence of PAM (6 μM), TRAP-6 induced aggregation was further inhibited 10-fold and collagen 4-fold further, highlighting the synergistic relationship between the three mediators.

Selecting inhibitory concentrations of PGI_2 (1 nM) and DEA/NONOate (100 nM), I further explored the effects of full $P2Y_{12}$ receptor inhibition with PAM (6 μM) with both PGI_2 and NO individually and in combination on platelet aggregation and then also the additional effects of aspirin on the powerful three-way synergy that was observed between the aforementioned mediators. Following incubation with PAM (6 μM), aggregation was significantly reduced by both PGI_2 and NO alone. However, much stronger inhibition was observed in the presence of the combination for both agonists tested, collagen (4 μg/ml) and TRAP-6 (25 μM). Interestingly, the addition of aspirin did not further reduce aggregation in the presence of PAM (6 μM) and PGI_2+NO following collagen stimulation. In TRAP-6 stimulated samples aspirin did not have any additional inhibitory action to that of PAM (6 μM) in the presence of PGI_2 or NO individually or in combination. Aggregation in response to collagen was reduced by PGI_2+NO and aspirin therapy alone, but there was no reduction in the response to TRAP-6 with no evidence of the powerful three-way synergy that was observed between $P2Y_{12}$ blockade and these inhibitors. These data support the hypothesis that the level of platelet inhibition *in vivo* will be dramatically affected by the presence and activity of these endothelially produced endogenous platelet inhibitors in the presence of $P2Y_{12}$ receptor antagonist therapy.

Next, continuing with an *in vitro* approach, I modelled events in the presence of suboptimal levels of $P2Y_{12}$ receptor blockade by using concentrations of PAM that were 50% and 25% of the effective concentration. Under these conditions it was noted that relative to consensus levels of DAPT there was no significant reduction in platelet aggregation. Notably, however, in the presence of NO and PGI_2 effective levels of inhibition were achieved, even when platelets were exposed to only 25% of the effective concentration of PAM. As expressed in heat maps, there is a clear interaction between

$P2Y_{12}$ blockade and the endothelial mediators that move platelets from reactive ('red') to unreactive ('green'). Interestingly, these comparisons indicate that 25% of the effective concentration of PAM plus NO and PGI_2 produces a stronger inhibition in LTA, the 'gold standard test', than 100% of the effective concentration of PAM in the absence of NO and PGI_2 (i.e. the normal conditions for testing *ex vivo* platelet responsiveness). Similarly, while NO, PGI_2 or $P2Y_{12}$ blockade alone had relatively little effect upon platelet granule release, determined as ATP release, when combined they caused more than 50% inhibition. These results indicate that even in the presence of effective $P2Y_{12}$ blockade, the presence of NO and PGI_2 lead to very much higher levels of platelet inhibition. Again, even in the presence of only partial 25% and 50% $P2Y_{12}$ receptor blockade, the addition of the combination of PGI_2 and NO caused more effective platelet inhibition than 100% of the effective concentration of PAM in the absence of these endothelial inhibitors. This suggests that in individuals in whom suboptimal $P2Y_{12}$ inhibition is achieved, such as poor clopidogrel metabolisers, anti-platelet efficacy may be particularly sensitive to any changes in endothelial function.

The *in vitro* data also demonstrate that the triple synergy between $P2Y_{12}$ blockade, NO and PGI_2 can be explained by changes in cAMP signalling, which is consistent with known interactions between NO and PGI_2 [322], and between PGI_2 and $P2Y_{12}$ [237]. The most pronounced increases in cAMP, but not cGMP, were seen with NO+PGI_2 together with $P2Y_{12}$ blockade following TRAP-6 stimulation. This increase in cyclic nucleotide activity was confirmed through measurement of VASP phosphorylation, a major substrate for cAMP- and cGMP-regulated protein kinases, which was increased following TRAP-6 stimulation in the presence of PAM in all cases but markedly so in the presence of PGI_2 and also DEA/NONOate+PGI_2.

In this first chapter I show that the endothelial inhibitors PGI_2 and NO influence the efficacy of $P2Y_{12}$ receptor blockade in inhibiting platelet function. Whilst PGI_2 and NO had little effect on their own, they caused almost complete inhibition of platelets treated with PAM. While NO and PGI_2 were added exogenously they are surrogates for the effects of endogenous NO and PGI_2 and other elevators of platelet cyclic nucleotides such as adenosine. This initial data suggests that following standard DAPT the level of platelet reactivity is a function of the level of $P2Y_{12}$ receptor blockade and the levels of NO and PGI_2 and *in vivo* platelet function is a product of both internal platelet responsive signalling reactivity and the external influence of the endothelium. Having demonstrated this is *in vitro,* in the next chapter I will further investigate these interactions by testing platelets *ex vivo* from healthy volunteers treated with standard anti-platelet therapies.

Chapter 4: The efficacy of $P2Y_{12}$ receptor antagonist therapy is strongly determined by endothelial mediators in healthy volunteers *ex vivo*

4.1 Introduction

In the previous chapter I described a potent three way synergy between $P2Y_{12}$ receptor blockade and the endothelial mediators, NO and PGI_2. A synergy much more powerful than those previously described between NO and PGI_2 [74], and both NO and PGI_2 individually with $P2Y_{12}$ blockade [238] [237]. I outlined that the inhibitory effects of both NO and PGI_2 are powerfully potentiated by $P2Y_{12}$ receptor blockade *in vitro* which, is suggestive that *in vivo* platelet function is a product of intrinsic platelet reactivity, modifiable by DAPT and the endogenous inhibitory endothelial mediators NO and PGI_2. This implies that for individual patients endothelial mediator production is an important determinant of the efficacy of DAPT, and that for individual patients *in vitro* measures of platelet reactivity do not accurately predict the *in vivo* effectiveness of DAPT due to the confounding of differences in endothelial mediator production. Here, my studies continue to test the hypothesis that within the circulation levels of endothelium-derived mediators are an important determinant of the efficacy of DAPT by adding exogenous NO and PGI_2 to standard *ex vivo* tests of platelet function in blood taken from healthy volunteers receiving anti-platelet therapies.

4.2 Methods

4.2.1 Study participants

24 healthy, non-smoking male volunteers (aged 18-40 years) were recruited and participated in the study. Health status was determined though medical history and physical examination, including blood pressure, pulse rate, blood chemistry and urinalysis. Volunteers with normal clinical profiles were included in the study.

4.2.2 Study protocol

Healthy volunteers abstained from aspirin, NSAIDs and any other anti-platelet therapy for 14 days before commencing the study. The volunteers were divided into three groups of 8. The first group received aspirin (75 mg, o.d.), the second prasugrel (10 mg, o.d.) and the third both aspirin (75 mg, o.d.) and prasugrel (10 mg, o.d.), to represent DAPT, for 7 days. Adherence was assessed by interview at each study visit. Blood and urine samples were collected before and after drug treatment on day 0 and day 7.

4.2.3 Blood collection and preparation of PRP/PPP

Blood was collected by venepuncture as described in section 2.2.3 and PRP/PPP was prepared as described in section 2.2.4.

4.2.4 LTA

Baseline aggregations to AA (1 mM), ADP (5 and 20 µM), Horm collagen (0.4, 4 and 10 µg/ml), U46619 (10 µM) and TRAP-6 (25 µM) were determined, as described in section 2.2.7.1. Aggregations to TRAP-6 (25 µM) and Horm collagen (4 µg/ml) were measured after pre-incubation of PRP with vehicle (NaOH 0.01 M,) DEA/NONOate (100 nM) or PGI_2 (1 nM) individually or in combination with DEA/NONOate and PGI_2 for 1 minute at 37 °C.

4.2.5 ADP + ATP Release

ADP + ATP release from platelets was assessed by lumi-aggregometry, as described in section 2.2.8. PRP was pre-incubated with vehicle or DEA/NONOate and/or PGI_2 as described above. ADP + ATP secretion was evaluated by luminescence in the presence of Chrono-Lume reagent after stimulation with collagen (4 µg/ml) or TRAP-6 (25 µM).

4.2.6 Flow cytometry

PRP was incubated with PGI_2 and/or DEA/NONOate or vehicle and then activated with TRAP-6 (25 µM). P-selectin and PAC-1 expression were measured using flow cytometry, as described in section 2.2.9.

4.2.7 cAMP and cGMP measurements

PRP was stimulated with collagen (4 µg/mL) or TRAP-6 (25 µM) in the presence of PGI_2 and/or DEA/NONOate or vehicle. After 4 min, platelets were lysed with Triton-X-100 (0.625%) and treated with iso-butylmethylxanthine (IBMX; 500 µM) and potassium fluoride (0.5 M). cAMP and cGMP concentrations were determined by HTRF-based competitive immunoassays.

4.2.8 Heat map generation and scaling

The heat maps were created in Microsoft excel. Conditional formatting was applied to the cells containing the data range. A colour scale was chosen to allow visual representation of the underlying data. At one end of the scale, red represents 100% aggregation of platelets or maximal platelet ATP release. On the other end of the scale, green represents 0% platelet aggregation or no ATP release. There is a gradual change in the colour scale from red to dark red to brown to dark green through to green

corresponding to decreasing percentage values. Dark brown represents the mid-way point (50%. The colour gradient was applied to the range of cells containing the data thus indicating where each data point falls within the data set. This allows a quick and powerful visual representation of my data alongside the other graphical resources and written text. The red (100%) and green (0%) are immediately differentiated and data points in between can be judged in conjunction with the underlying data and scale represented below.

The colour scale is as follows:

4.3 Results

4.3.1 LTA responses to standard agonists following monotherapy aspirin or prasugrel and DAPT

In baseline studies, high pre-treatment levels of platelet reactivity were observed, with final aggregation values of over 60% for all agonists tested, as is normal in naïve healthy volunteers [244, 323, 324]. Following treatment with aspirin or prasugrel there was powerful inhibition of platelet responses to AA or ADP, respectively, (see below) in keeping with the mode of action of these agents [244, 324].

In individuals taking aspirin, standard LTA responses to AA (1 mM) were strongly inhibited (74±7% to 2±1%), as were responses to collagen (0.4 µg/ml). Responses to ADP (5 µM) were also significantly reduced although to a lesser degree (74±6 to 46±8%), while those to U46619 (10 µM) were unaffected (Figure 4.1A). In individuals taking prasugrel, aggregatory responses to AA (67±5% to 12±6%), collagen (0.4 µg/ml) (69±2% to 17±4%), ADP (5 µM) (70±2% to 2±1%) and U46619 (72±2% to 50±5%) were all significantly reduced (Figure 4.1B). Aggregations induced by AA, collagen (0.4 µg/ml) and ADP (5 µM and 20 µM) were abolished in individuals taking DAPT (aspirin plus prasugrel) while responses to U46619 (71±1% to 29±10%) and collagen (10 µg/ml) (69±2% to 44±3%) were strongly reduced (Figure 4.1C).

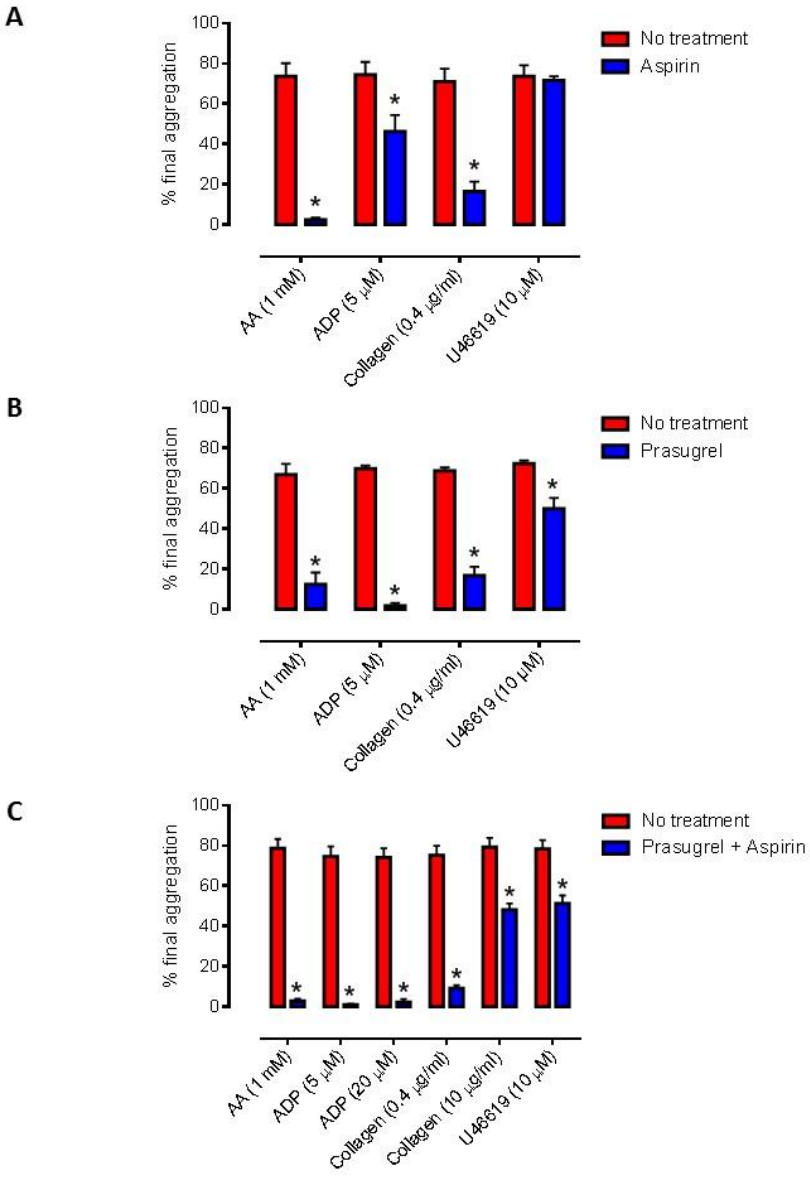


Figure 4.1: Standard platelet aggregation tests. Standard LTA responses to AA (1 mM), ADP (5 and 20 µM), collagen (0.4 and 10 µg/mL) and U46619 (10 µM) in healthy volunteers before and following treatment with (A) aspirin (75 mg), (B) prasugrel (10 mg), or (C) DAPT (aspirin, 75 mg + prasugrel, 10 mg) for 7 days. N=8 for all. Significance is shown as * $p<0.05$ treated vs non-treated.

4.3.2 The individual and combined effects of PGI_2, NO and DAPT on platelet aggregation

Representative LTA traces are displayed in figure 4.2. In control PRP neither DEA/NONOate nor PGI_2 individually or in combination had an effect on platelet aggregation responses following collagen (4 µg/ml) or TRAP-6 (25 µM) stimulation (Figure 4.2.A/C).

Following DAPT treatment, collagen (4 µg/ml) induced aggregation was reduced for all conditions tested; vehicle, 70% to 39%; DEA/NONOate, 68% to 29%; PGI_2 70% to 21% and PGI_2+DEA/NONOate, 64% to 11%. In TRAP-6 (25 µM) stimulated PRP following DAPT, aggregation was minimally reduced in the presence of vehicle (75% to 61%) but more so in the presence of DEA/NONOate (76% to 52%) and PGI_2 (76% to 49%). The greatest reduction in platelet aggregation was observed in platelets exposed to the combination of PGI_2+DEA/NONOate (70% to 20%) (Figure 4.2.B/D).

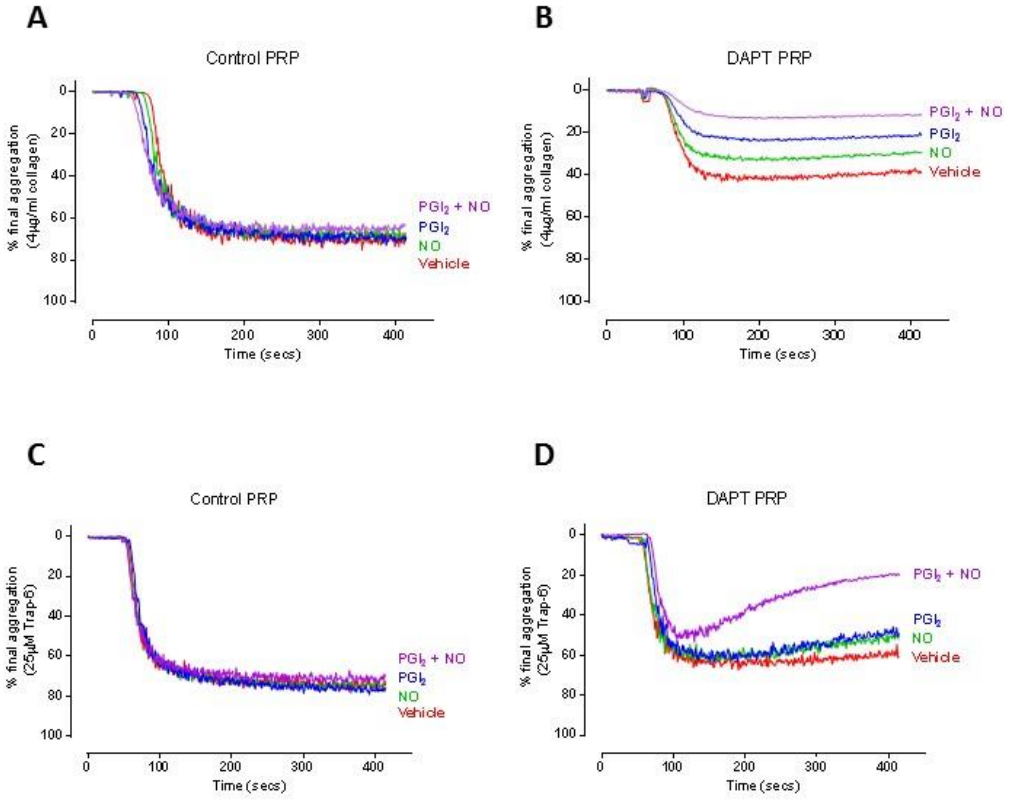


Figure 4.2: The effect of DAPT on platelet aggregation. Representative LTA traces of PRP before and after DAPT (aspirin, 75 mg + prasugrel, 10 mg) treatment in the presence of vehicle (NaOH, 0.01 M), DEA/NONOate (100 nM), PGI_2 (1 nM), or DEA/NONOate+PGI_2 following (A/B) collagen (4 µg/mL) or (C/D) TRAP-6 amide (25 µM) stimulation. Traces representative of n=1.

4.3.3 The individual and combined effects of PGI_2 and NO on aspirin or prasugrel monotherapy and DAPT on platelet aggregation following collagen stimulation

PRP from each therapy group was tested with the addition of PGI_2 and/or NO. Before therapy, PGI_2 (1 nM) or DEA/NONOate (100 nM) had little effect upon platelet aggregation in response to collagen (4 μg/ml), whilst NO+PGI_2 produced some inhibition (Figure 4.3.A/B/C). Following DAPT, aggregation in the presence of vehicle was significantly reduced from 73±2% to 31±2% ($p<0.05$; Figure 4.3.A), as seen previously with a lower concentration of collagen (0.4 μg/mL; Figure 4.1.C). Inhibition of platelet aggregation was increased by the addition of either PGI_2 (14±2%) or DEA/NONOate (17±3%), but further still by the combination of PGI_2+DEA/NONOate (5±1%) (Figure 4.3.A). A similar pattern, i.e. the highest levels of platelet inhibition were seen with the addition of PGI_2+DEA/NONOate post therapy, were observed in both aspirin and prasugrel monotherapy treated groups (Figure 4.3.B/C). Of note, in these PGI_2+DEA/NONOate treated samples the highest net reduction in platelet aggregation was noted following DAPT (Native PRP: 56±6% to post DAPT: 5±1%, net reduction: 51%), as compared to that observed for prasugrel (Native PRP: 28±8% to post prasugrel: 1±1%, net reduction: 27%) and the smallest net reduction following aspirin therapy (Native PRP: 16±6% to post aspirin 2±1%, net reduction 14%) (Figure 4.3.A/B/C).

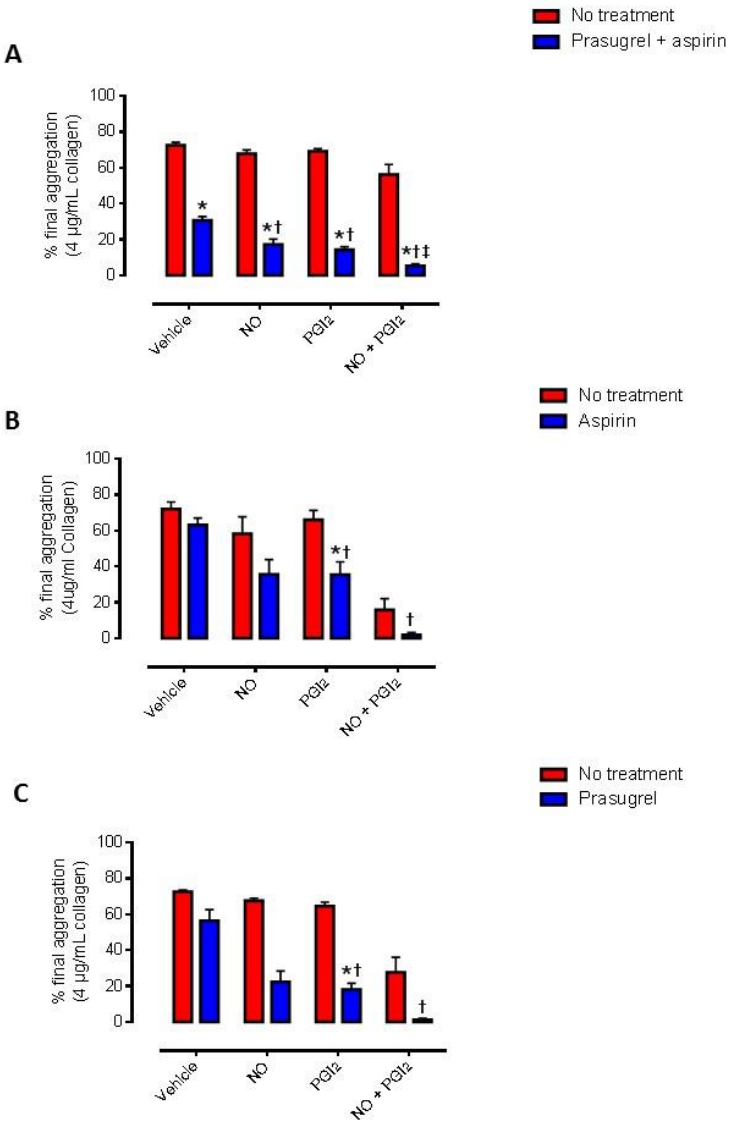


Figure 4.3: Interactions of NO and PGI_2 with DAPT, aspirin and prasugrel: platelet aggregation following collagen stimulation. Bar graphs of platelet aggregation in response to collagen (4 µg/ml). Aggregometry was conducted before and after 7 days of DAPT (aspirin, 75 mg + prasugrel, 10 mg), aspirin (75mg) or prasugrel (10mg). Aggregometry was conducted in the presence of vehicle (NaOH, 0.01 M), NO (100 nM), PGI_2 (1 nM), or NO + PGI_2. Data are presented as final aggregation (%, mean±SEM). 3 data set comparisons have been performed. Significance is shown as * $p<0.05$ for pre- (red) versus post- (blue) anti-platelet therapy. Significance is shown † $p<0.05$ post- (blue) anti-platelet therapy, vehicle versus inhibitor treatment. Significance is shown ‡ $p<0.05$ post- (blue) anti-platelet therapy vs post therapy PGI_2-treated. N=8 for all.

4.3.4 The individual and combined effects of PGI_2 and NO on aspirin or prasugrel monotherapy and DAPT following TRAP-6 stimulation

The addition of PGI_2, DEA/NONOate or DEA/NONOate+PGI_2 had negligible effects upon platelet aggregation induced by TRAP-6 (25 μM) in native PRP (Figure 4.4). However, TRAP-6 (25 μM)-induced aggregation was significantly reduced by DAPT in the presence of vehicle, PGI_2 or DEA/NONOate (74±3% to 57±4%, 70±3% to 48±6% and 71±3% to 49±6%, respectively). Whilst when DEA/NONOate+PGI_2 was present in the presence of DAPT it was much reduced still further (67±3% to 19±6%, $p<0.05$; Figure 4.4.A). A similar pattern was observed in the prasugrel monotherapy group, in which prasugrel significantly reduced platelet aggregation ($p<0.05$) in the presence of either PGI_2 (70±1% to 43±6%) or DEA/NONOate (72±1% to 50±5%) but the highest levels of inhibition were observed with the addition of PGI_2+DEA/NONOate (63±3% to 7±3%) (Figure 4.4.C). This pattern was not noted in the aspirin monotherapy group where aspirin treatment only moderately reduced platelet aggregation significantly in the presence of PGI_2+DEA/NONOate (69±5% to 28±9%). Aspirin therapy did not reduce platelet aggregation in the presence of either PGI_2 or DEA/NONOate alone (Figure 4.4.B).

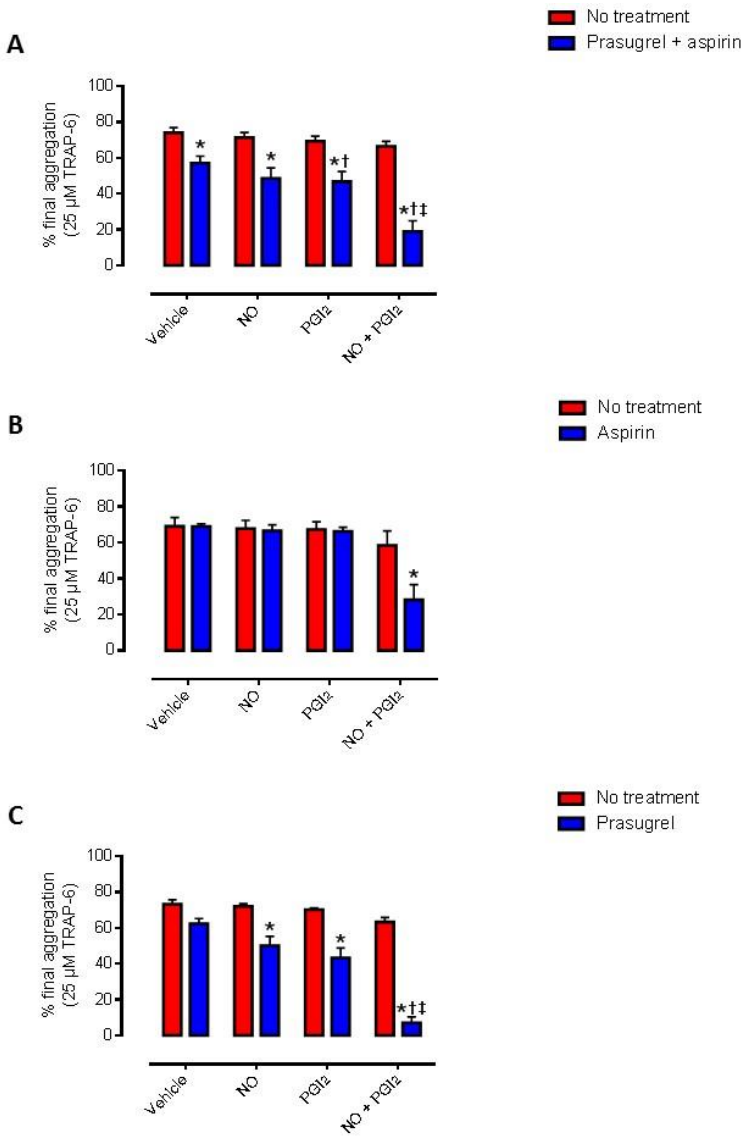


Figure 4.4: Interactions of NO and PGI_2 with DAPT, aspirin and prasugrel: platelet aggregation following TRAP-6 stimulation. Bar graphs of platelet aggregation in response to TRAP-6 (25 µM). Aggregometry was conducted before and after 7 days of DAPT (aspirin, 75 mg + prasugrel, 10 mg), aspirin 75mg or prasugrel 10mg. Aggregometry was conducted in the presence of vehicle (NaOH, 0.01 M), NO (100 nM), PGI_2 (1 nM), or NO + PGI_2. Data are presented as final aggregation (%, mean±SEM). 3 data set comparisons have been performed. Significance is shown as * $p<0.05$ for pre- (red) versus post- (blue) anti-platelet therapy. Significance is shown † $p<0.05$ post- (blue) anti-platelet therapy, vehicle versus inhibitor treatment. Significance is shown ‡ $p<0.05$ post- (blue) anti-platelet therapy vs post therapy PGI_2-treated. N=8 for all.

4.3.5 Heat maps displaying the effects of PGI_2 and NO on platelet aggregation following aspirin or prasugrel monotherapy and DAPT

To make data readily accessible results are presented in the form of heat maps that move from red to green, indicating movement from full platelet aggregation in red to

no platelet activation in green. These heat maps express a clear interaction between anti-platelet therapies and PGI_2 and NO that move platelets from reactive ('red') to unreactive ('green'). This is observable for both primary agonists tested, collagen (4 µg/ml) (Figure 4.5.A) and TRAP-6 (25 µM) (Figure 4.5.B). Though a weak effect of this transition is observed following aspirin therapy the effects are much more marked following both prasugrel monotherapy and DAPT. In the context of $P2Y_{12}$ receptor blockade through DAPT and prasugrel therapy, both PGI_2 and NO alone reduced platelet aggregation although it is the combination of the two which inhibited aggregation most strongly. Aspirin therapy only decreased aggregation when combined with the combination of NO+PGI_2, with these effects being most evident in the collagen (4 µg/ml) as opposed to TRAP-6 (25 µM) stimulated platelets.

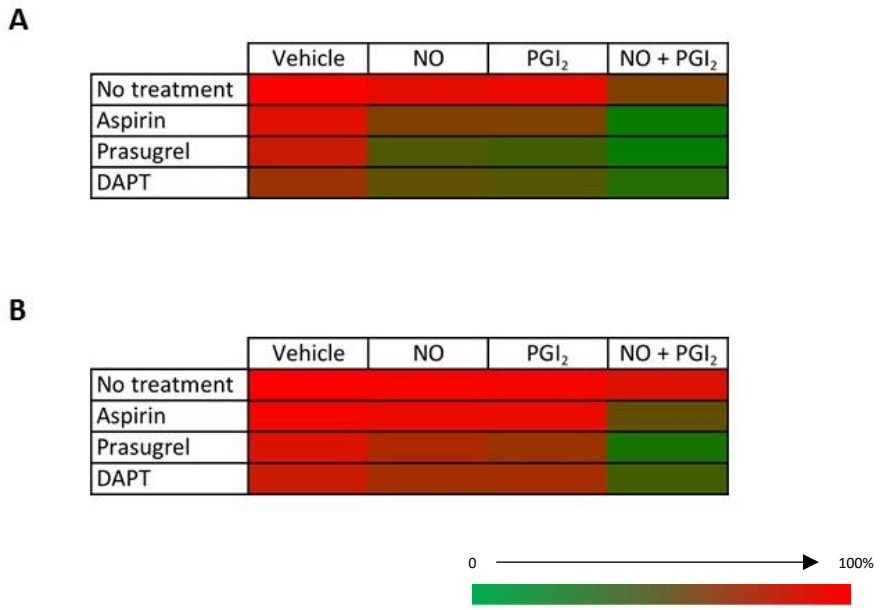


Figure 4.5: Heatmaps representing interactions of NO and PGI_2 with aspirin, prasugrel and DAPT. Heatmaps of platelet aggregation in response to (A) collagen (4 µg/ml), and (B) TRAP-6 amide (25 µM). Aggregometry was conducted before and after 7 days of aspirin (75 mg), prasugrel (10 mg) or DAPT (aspirin, 75 mg + prasugrel, 10 mg) in the presence of vehicle (NaOH, 0.01 M), NO (100 nM), PGI_2 (1 nM), or NO + PGI_2. Data are presented as heatmaps indicating maximum final aggregation with red and minimum final aggregation with green. N = 8 for all.

4.3.6 The individual and combined effects of PGI_2, NO and DAPT on ADP + ATP release

To further characterise the effects of PGI_2 and NO on platelets, ADP + ATP release in the lumi-aggregometer was determined as a measure of dense granule secretion. Representative lumi-aggregometry traces are displayed below in figure 4.6. In native PRP there was a reduction in ATP release only in the presence of PGI_2+DEA/NONOate. DAPT alone did not cause a reduction in ATP release, however the addition of either DEA/NONOate or PGI_2 did reduce ATP release. The lowest ATP release was observed following DAPT in the presence of DEA/NONOate+PGI_2.

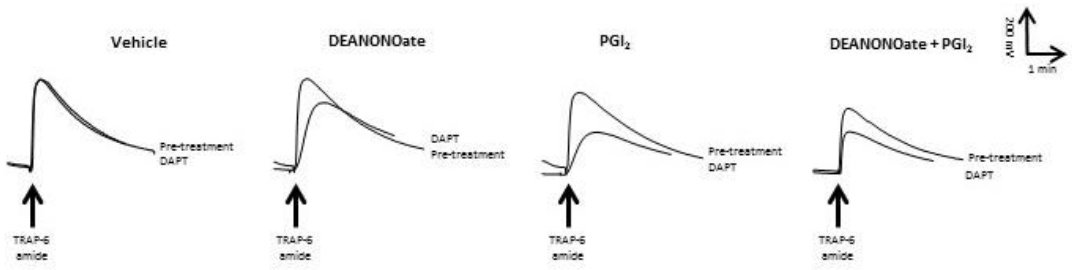


Figure 4.6: The effect of DAPT on platelet ATP release. Representative lumi-aggregometry traces before and after DAPT (aspirin, 75 mg + prasugrel, 10 mg) treatment in the presence of vehicle (NaOH, 0.01 M), DEA/NONOate (100 nM), PGI_2 (1 nM), or DEA/NONOate+PGI_2 following TRAP-6 amide (25 µM) stimulation. Traces representative of n=1.

4.3.7 The individual and combined effects of PGI_2 and NO together with aspirin or prasugrel monotherapy and DAPT on ADP + ATP release

In native PRP, PGI_2+DEA/NONOate caused variable levels of collagen-induced ATP release in the three groups tested; ATP release in the DAPT group was not affected by the addition of PGI_2+DEA/NONOate (3.8±0.8 to 3.5±0.8 nM) however, in the aspirin and prasugrel monotherapy groups ATP release was reduced (6.8±0.9 to 2.4±0.9 nM) and (8.6±0.5 to 3.1±0.4 nM) by PGI_2+NONOate (Figure 4.7.A/C/D). DAPT reduced ATP release with the addition of PGI_2 (3.8±0.8 to 0.9±0.15 nM, $p<0.05$) (Figure 4.7.A). Aspirin therapy also significantly reduced ATP release in the presence of PGI_2 (Figure 4.7.C). Prasugrel

therapy significantly reduced ($p<0.05$) ATP release in all conditions tested. ATP was also significantly reduced ($p<0.05$) by DEA/NONOate and PGI_2 individually and in combination compared to vehicle (vehicle, 8.6±0.5 to 5.2±0.4; DEA/NONOate, 7.3±0.5 to 1.4±0.2; PGI_2, 6.6±0.3 to 0.9±0.15 and PGI_2+NO 3.1±0.4 to 1±0.3 nM) (Figure 4.7.D). In platelets stimulated with TRAP-6 (25 µM), ATP release was not affected by DAPT in the presence of vehicle (pre-treatment, 7.5±1.7; post-treatment, 7.7±0.6 nM) or by the addition of PGI_2 and/or DEA/NONOate (Figure 4.7.B).

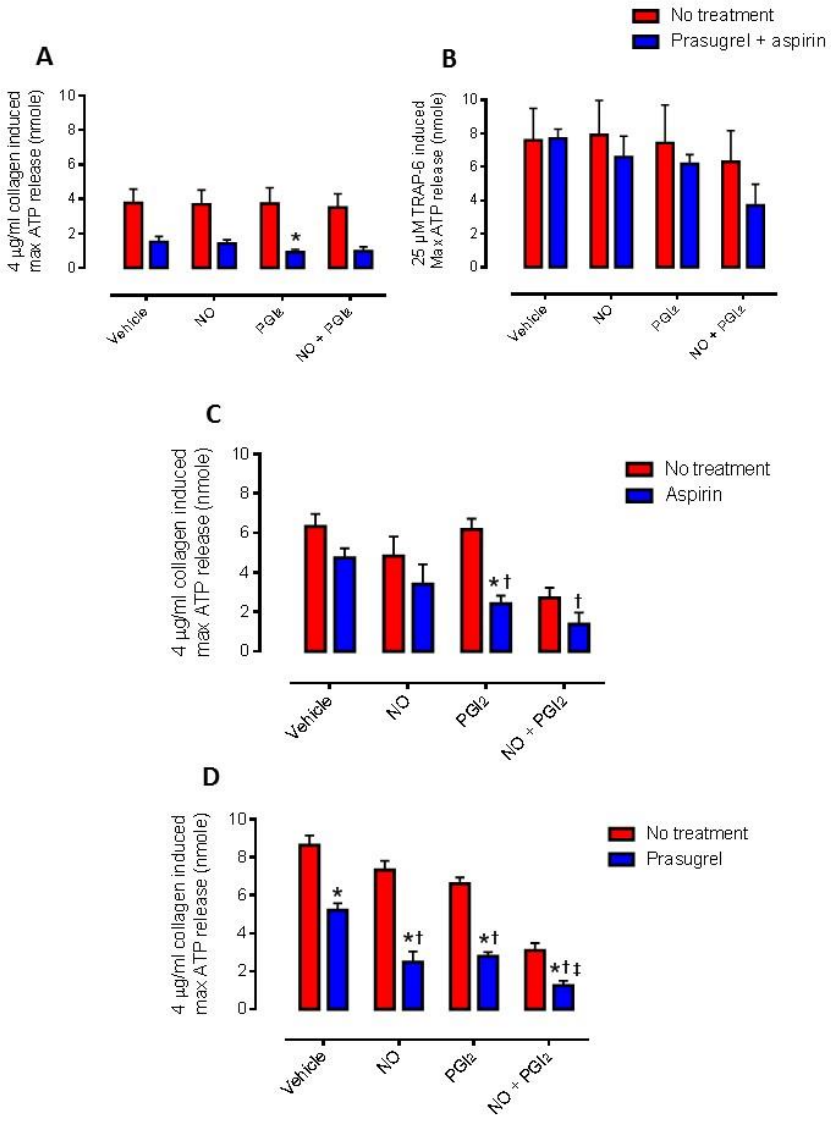


Figure 4.7: Interactions of NO and PGI_2 with DAPT, aspirin and prasugrel: platelet ADP/ATP release. Bar graphs of platelet ADP/ATP release in response to (A/C/D) collagen (4 µg/ml), and (B) TRAP-6 amide (25 µM). Lumi-aggregommetry experiments were conducted before and after 7 days of DAPT (aspirin, 75 mg + prasugrel, 10 mg), aspirin (75mg) or prasugrel (10mg), in the presence of vehicle (NaOH, 0.01 M), NO (100 nM), PGI_2 (1 nM), or NO + PGI_2. Data are presented

as ATP release (nmole, mean±SEM). 3 data set comparisons have been performed. Significance is shown as * p<0.05 for pre- (red) versus post- (blue) anti-platelet therapy. Significance is shown † p<0.05 post- (blue) anti-platelet therapy, vehicle versus inhibitor treatment. Significance is shown ‡ p<0.05 post- (blue) anti-platelet therapy vs post therapy PGI_2-treated. N=8 for all.

4.3.8 Heat maps displaying the effects on ATP release of PGI_2 and NO in control conditions or in the presence of aspirin or prasugrel monotherapy and DAPT

As previously, the results in 4.3.7 are represented in the form of heat maps transitioning from red to green to indicate movement from high platelet ATP release in red to low platelet ATP release in green. A transition of red to green was observed with the addition of PGI_2, NO and PGI_2+NO following treatment with all three anti-platelet therapy regimes in platelets stimulated with collagen (4 µg/ml; Figure 4.8.A) or TRAP-6 (25 µM; Figure 4.8.B).

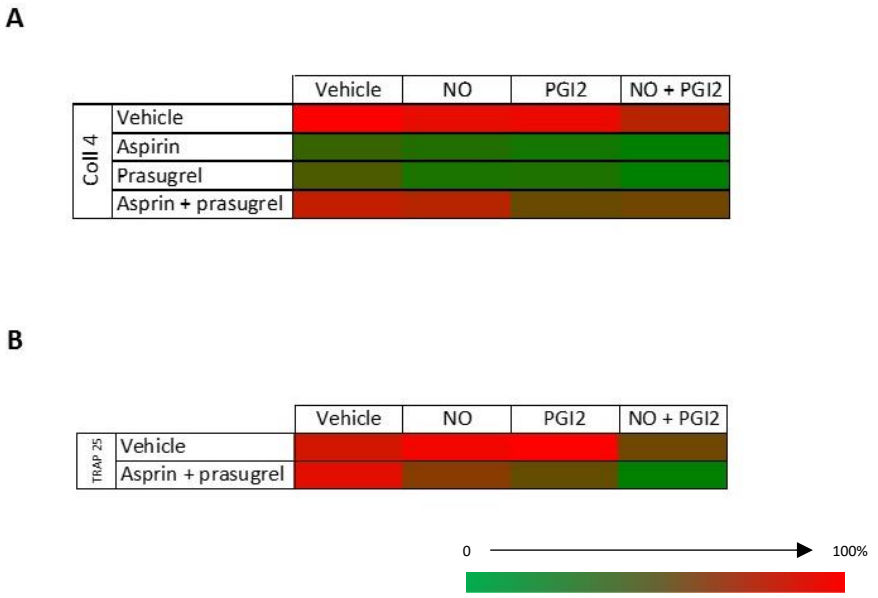


Figure 4.8: Heat maps representing interactions of NO and PGI_2 with aspirin, prasugrel and DAPT. Heat maps of platelet ATP release in response to (A) collagen (4 µg/ml), and (B) TRAP-6 amide (25 µM). Lumi-aggregometry was conducted before and after 7 days DAPT (aspirin, 75 mg + prasugrel, 10 mg), aspirin (75 mg) or prasugrel (10mg) in the presence of vehicle (NaOH, 0.01 M), NO (100 nM), PGI_2 (1 nM), or NO + PGI_2. Data are presented as heat maps indicating maximum ATP release with red and minimum ATP release with green. N = 8 for all.

4.3.9 P-selectin expression and GPIIb/IIIa activation in the presence of PGI_2 and NO together with aspirin or prasugrel monotherapy and DAPT

To further characterise the effects of PGI_2 and NO on platelets, PAC-1 and P-selectin expression were determined. Representative histograms of the effects of DAPT and endothelial inhibitors are displayed below. In native PRP, there was a reduction in PAC-1 and P-selectin expression in the presence of PGI_2 and PGI_2+DEA/NONOate. DAPT reduced PAC-1 and P-selectin expression in all conditions tested (Figure 4.9).

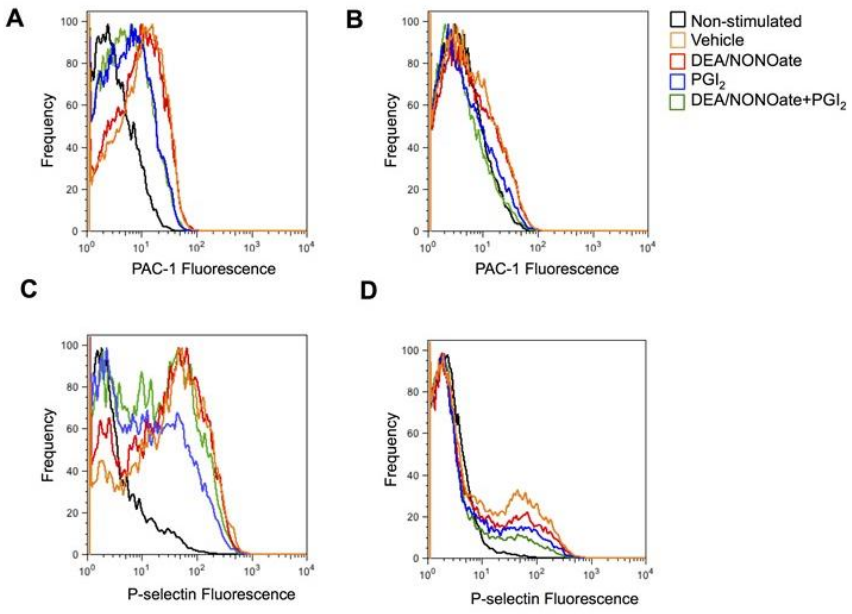


Figure 4.9: Representative histograms of the effects of DAPT on platelet P-selectin and GPIIb/IIIa expression. GPIIb/IIIa activation by PAC-1 binding in the **(**A**)** absence and **(**B**)** presence of DAPT and P-selectin expression in the **(**C**)** absence and **(**D**)** presence of DAPT as measured by flow cytometry in PRP stimulated with TRAP-6 (25 µM) in the presence of vehicle (NaOH, 0.01 M), DEA/NONOate (100 nM), PGI_2 (1 nM), or DEA/NONOate + PGI_2.

In native PRP, TRAP-6 induced expression of P-selectin and GPIIb/IIIa produced a similar pattern in all 3 volunteer groups with mild reductions caused by PGI_2 and DEA/NONOate individually, and moderate reductions caused by PGI_2+DEA/NONOate (Figure 4.10). Both P-selectin expression and GPIIb/IIIa activation following DAPT were strongly reduced in all conditions tested. For both markers, PGI_2+DEA/NONOate further significantly reduced expression ($p<0.05$) compared to vehicle treated PRP (Figure 4.10.A/B). Aspirin therapy caused no significant reductions in either P-selectin or GPIIb/IIIa activation

(Figure 4.10.C/D). In the presence of prasugrel therapy, P-selectin expression was reduced in the vehicle treated group (25±6 MFI to 9±3 MFI). GPIIb/IIIa activation was significantly reduced by prasugrel in all conditions tested and also further by DEA/NONOate, PGI_2 and PGI_2+DEA/NONOate: vehicle, 19±4 to 1.6±0.3 MFI; DEA/NONOate, 10±2 to 0.4±0.2 MFI; PGI_2, 13±2 to 0.5±0.2 MFI and NO+PGI_2, 5±1 to 0.2±0.2 MFI (Figure 4.10.E/F).

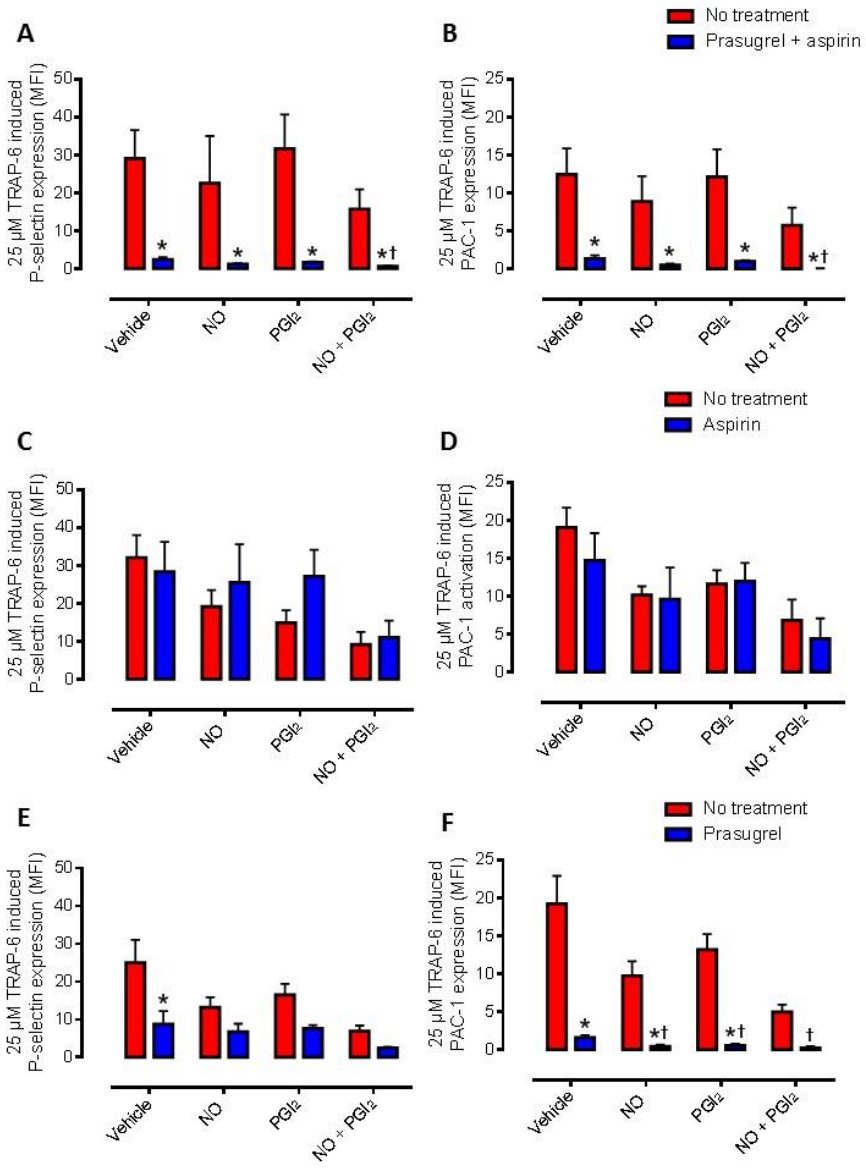


Figure 4.10: Interactions of NO and PGI_2 with DAPT, aspirin and prasugrel: platelet P-selectin and GPIIb/IIIa expression. Bar graphs of P-selectin expression (A/C/E) and PAC-1 activation (B/D/F) stimulated with TRAP-6 amide (25 µM). Flow cytometry experiments were conducted before and after 7 days of DAPT (aspirin, 75 mg + prasugrel, 10 mg), aspirin 75mg or prasugrel

10mg. Flow cytometry was conducted in the presence of vehicle (NaOH, 0.01 M), NO (100 nM), PGI_2 (1 nM), or NO + PGI_2. Data are presented as mean fluorescence index (MFI, mean±SEM). 3 data set comparisons have been performed. Significance is shown as * $p<0.05$ for pre- (red) versus post- (blue) anti-platelet therapy. Significance is shown † $p<0.05$ post- (blue) anti-platelet therapy, vehicle versus inhibitor treatment. Significance is shown ‡ $p<0.05$ post- (blue) anti-platelet therapy vs post therapy PGI_2-treated. N=8 for all.

4.3.10 Heat maps displaying the effects on P-selectin and GPIIb/IIIa expression of PGI_2 and NO in the presence of aspirin or prasugrel monotherapy and DAPT

In native PRP, predominantly red cells are present in the matrix representing high levels of platelet activation. With the addition of PGI_2+DEA/NONOate there is a weak transition to green. These results are largely mirrored in the aspirin treated group for both ADP and TRAP-6. Results for P-selectin (Figure 4.11.A) and GPIIb/IIIa expression (Figure 4.11.B) in the DAPT and prasugrel treated groups are markedly different, with all cells in the matrix displaying green representing high levels of platelet inhibition for both ADP and TRAP-6.

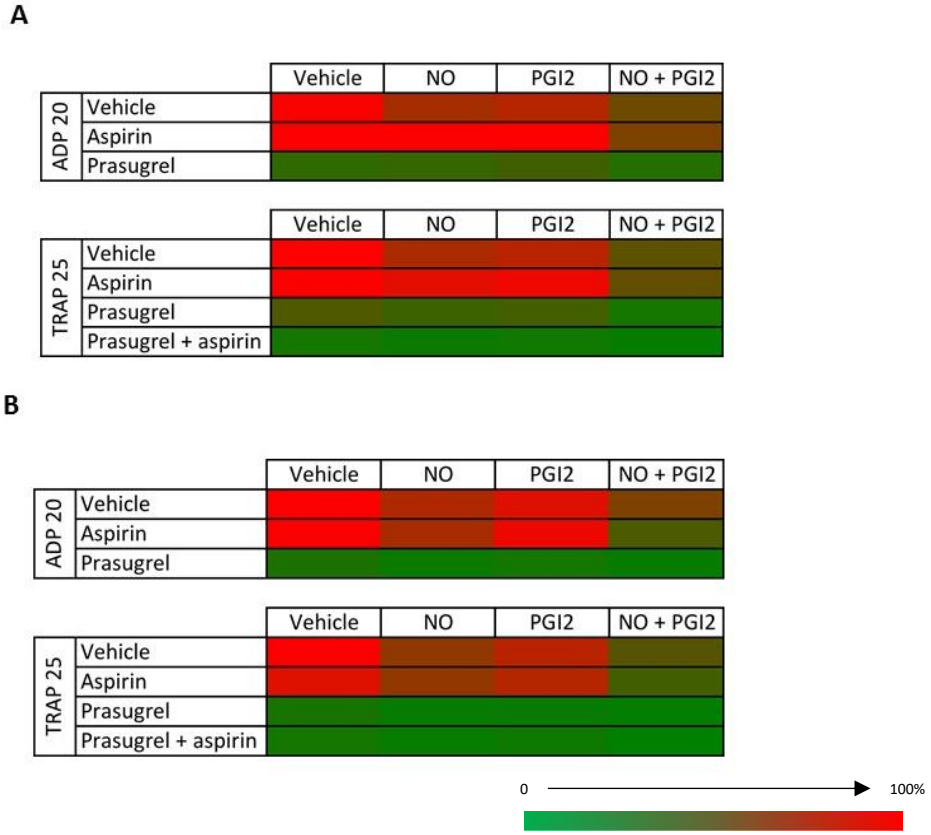


Figure 4.11: Heat maps representing interactions of NO and PGI_2 with aspirin, prasugrel and DAPT. Heat maps of (A) P-selectin expression and (B) PAC-1 activation in response to ADP (20 µM) and TRAP-6 amide (25 µM). Flow cytometry was conducted before and after 7 days DAPT

(aspirin, 75 mg + prasugrel, 10 mg), aspirin or prasugrel in the presence of vehicle (NaOH, 0.01 M), NO (100 nM), PGI_2 (1 nM), or NO + PGI_2. Data are presented as heat maps indicating maximum MFI with red and MFI with green. N = 8 for all.

4.3.11 Involvement of cAMP and cGMP in the synergistic effects of $P2Y_{12}$ blockade, PGI_2 and NO

I found no significant changes in cGMP levels in platelets in response to DEA/NONOate+PGI_2 following treatment with DAPT (Figure 4.12.C/D). In collagen-stimulated platelets following DAPT, cAMP levels (0.42±0.12 nM) were significantly increased ($p<0.05$) by DEA/NONOate+PGI_2 (4.42±0.98 nM) (Figure 4.12.A). A significant increase ($p<0.05$) was also observed in platelets stimulated with TRAP-6 (0.34±0.1 to 2±0.35 nM) (Figure 4.12.B).

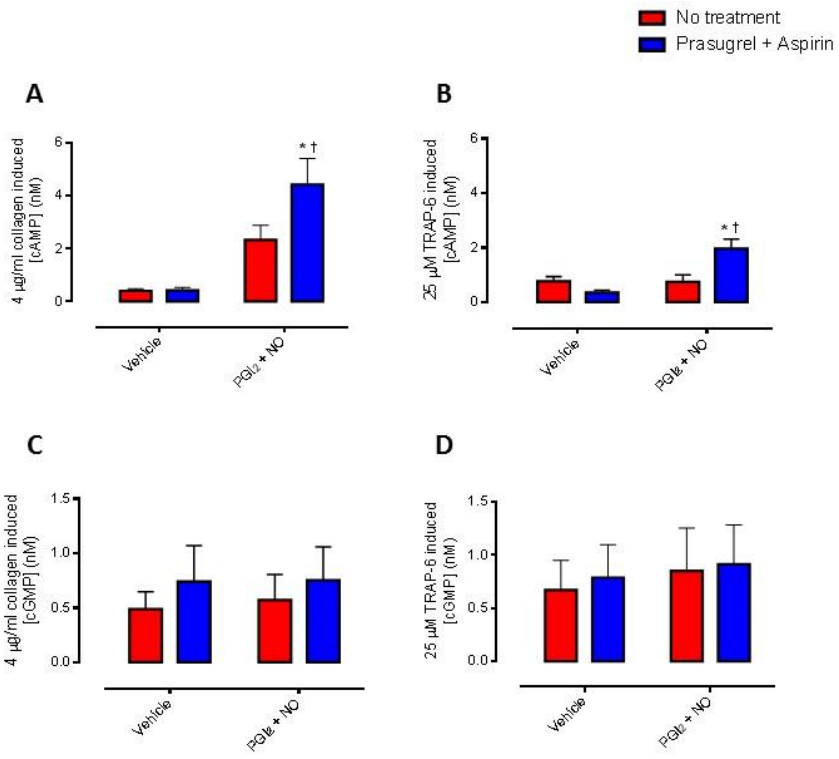


Figure 4.12: Interactions of NO + PGI_2 with DAPT with prasugrel (10 mg) plus aspirin (75 mg): platelet cyclic AMP and GMP release. Bar graphs of cAMP release (A/B) following stimulation of platelets with (A) collagen (4 µg/ml) and (B) TRAP-6 amide (25 µM). Bar graphs of cGMP release (C/D) following stimulation with (C) collagen (4 µg/ml) and (D) TRAP-6 amide (25 µM). Experiments were conducted before and after 7 days of DAPT (aspirin, 75 mg + prasugrel, 10 mg) in the presence of vehicle (NaOH, 0.01 M) or NO + PGI_2 (100 nM + 1 nM). Data are presented

as nanomolar (nM, mean±SEM). Significance is shown as * p<0.05 vs non-treated, † p<0.05 vs NaOH-treated. N=8 for all experiments.

To further validate these results, cyclic nucleotide levels were also measured in platelets from healthy volunteers pre and post treatment with another $P2Y_{12}$ antagonist, ticagrelor. Similarly to the results obtained for DAPT with prasugrel plus aspirin, DAPT with ticagrelor plus aspirin did not alter the cGMP response in either collagen-stimulated (4 µg/ml) or TRAP-6 (25 µM) stimulated platelets. There was also no change in cGMP formation following the addition of PGI_2+DEA/NONOate (Figure 4.13.C/D). In contrast, in collagen-stimulated platelets the combination of PGI_2+DEA/NONOate significantly elevated cAMP levels following treatment with aspirin plus ticagrelor (0.32±0.13 nM to 3.51±1.13 nM, p<0.05) (Figure 4.13.A).

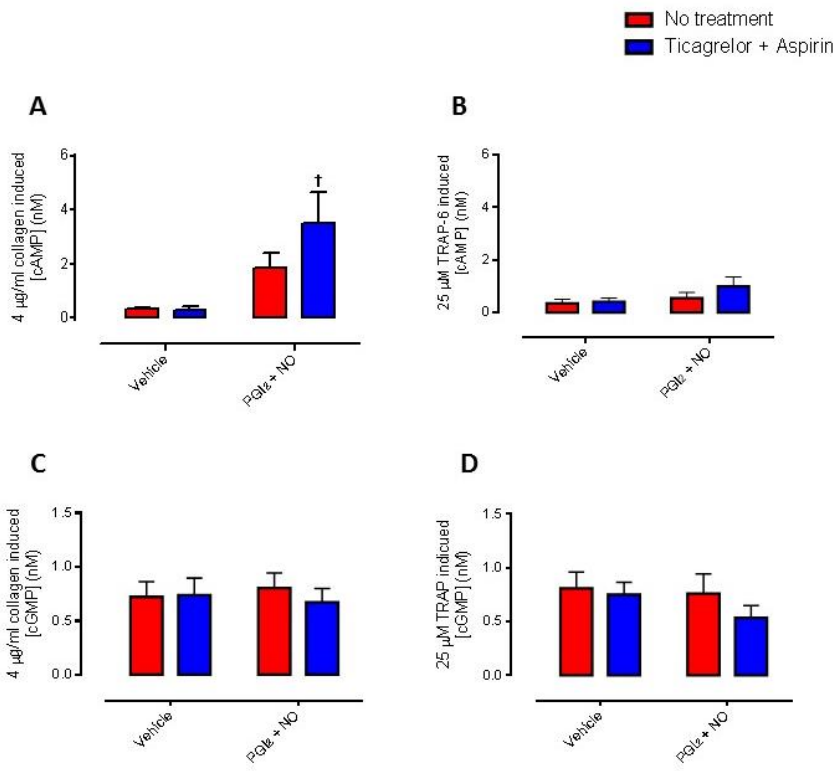


Figure 4.13: Interactions of NO + PGI_2 with DAPT with ticagrelor (90 mg) and aspirin (75 mg): platelet cyclic AMP and GMP release. Bar graphs of cAMP release (A/B) following stimulation with (A) collagen (4 µg/ml) and (B) TRAP-6 amide (25 µM). Bar graphs of cGMP release (C/D) following stimulation with (C) collagen (4 µg/ml) and (D) TRAP-6 amide (25 µM). Experiments were conducted before and after 7 days of DAPT (aspirin, 75 mg, plus ticagrelor, 90 mg) in the

presence of vehicle (NaOH, 0.01 M) or NO + PGI_2 (100 nM + 1 nM). Data are presented as nanomolar (nM, mean±SEM). Significance is shown as * $p<0.05$ vs non-treated, † $p<0.05$ vs NaOH-treated. N=8 for all.

4.4 Discussion

The data presented here show in healthy individuals receiving standard $P2Y_{12}$ receptor antagonist therapy producing consensus levels of platelet inhibition, that *ex vivo* responses to the strong primary platelet activators collagen and TRAP-6 are powerfully influenced by the presence of NO and PGI_2. It is well known that NO and PGI_2 synergise to inhibit platelets [74] and it has been demonstrated that $P2Y_{12}$ antagonists potentiate the inhibitory actions of both PGI_2, dependent upon cAMP [237], and NO, dependent upon cGMP generation [238]. In the studies presented here, through measures of aggregation, ATP release, activation of GP IIb/IIIa receptors and P-selectin expression, I demonstrate that these strong interactions between $P2Y_{12}$ receptor blockade and endothelial derived mediators produce profound inhibitory effects upon platelets tested *ex vivo*.

I firstly established that the drug regime given in these studies elicited satisfactory reduction in baseline reactivity therefore, establishing effectiveness of $P2Y_{12}$ and/or COX inhibition in accordance with suggested analytical cut-offs [264]. These reductions were against high pre-treatment levels of platelet reactivity (>70% response to 5 μM ADP) [123]. In these studies care was taken to include the standard measures of platelet function as determined in consensus statements [325]. Then to make data readily accessible, results were additionally presented in the form of heat maps that move from red to green, indicating movement from full platelet activation to no platelet activation.

In volunteers taking DAPT, it was noted that inhibition of LTA responses to ADP and AA were in keeping with consensus statements of effective DAPT; i.e. in this study the drugs

were working to an effective level of clinical efficacy. Despite this level of effective inhibition, high concentrations of the strong primary platelet activators, TRAP-6 or collagen, still caused notable platelet aggregation in all three groups. Addition of low concentrations of NO or PGI_2 individually had little effect on their own pre-treatment, nor did they affect aggregation to TRAP-6 following aspirin therapy. However, in the presence of $P2Y_{12}$ blockade by prasugrel or DAPT, the addition of NO or PGI_2 significantly reduced aggregation reflecting the known individual synergies between $P2Y_{12}$ blockade and PGI_2 and NO. Addition of low concentrations of NO and PGI_2 in combination, to model the environment within the blood vessel, had little effect on their own pre-treatment but led to almost complete inhibition of activation in platelets from individuals treated with DAPT (19±6) or prasugrel (7±3%), demonstrating the three-way synergy between these inhibitors and $P2Y_{12}$ blockade. The combination of NO+PGI_2 did reduce aggregation post-aspirin therapy but not to such a powerful degree (28±9%). PGI_2 and NO are known to synergise powerfully leading to platelet inhibition so we can well expect to see some inhibitory effect in platelets from individuals treated with aspirin. However, without the additional three way synergy of $P2Y_{12}$ receptor blockade this level of inhibition is far inferior to that seen in platelets from individuals treated with prasugrel.

With regard to collagen-induced aggregation, a similar pattern was observed for all three treatment groups post treatment, in that platelet aggregation was reduced individually by NO and PGI_2 but very much greater reductions were observed in the presence of the two inhibitors in combination. DAPT significantly reduced aggregation in all conditions tested and also in the presence of NO, PGI_2 and NO+PGI_2. These effects were stronger than those seen following monotherapy with aspirin or prasugrel alone

where aggregation was only significantly reduced with the addition of PGI_2 or PGI_2+NO. Of note, far greater net reductions in aggregation were observed in the DAPT (51%) and prasugrel (27%) groups in the presence of NO+PGI_2 as compared to aspirin (14%). These results are in keeping with the fact that DAPT has more powerful anti-platelet effects than aspirin or prasugrel alone, that collagen is a weaker platelet agonist than TRAP-6 and also a lower concentration of agonist was used so the inhibitory effects of PGI_2 and NO were more pronounced. Platelet activation was also assessed by collagen induced-ADP release where DAPT led to low levels of ATP release in all conditions tested. Prasugrel therapy alone also had a potent effect causing significant reductions in ATP release in all conditions tested. Notably, all three inhibitor combinations were significantly reduced compared to pre-treatment and also compared to vehicle. Following aspirin treatment, I observed a reduction in ATP release only in the presence of the combination of NO+PGI_2.

The interactions of PGI_2 and NO with either DAPT, aspirin or prasugrel therapies were also studied using flow cytometry by assessing both PAC-1 and P-selectin expression following TRAP-6 (25 μM) stimulation. For DAPT, both P-selectin and PAC-1 expression were very greatly reduced in all conditions tested. Notably, the addition of PGI_2+NO caused further significant decreases in the expression of both markers as compared to vehicle (P-selectin, 96% and PAC-1, 97%). Interestingly, aspirin therapy did not significantly reduce P-selectin or PAC-1 expression in any condition tested, or with the addition of NO and/or PGI_2 individually or in combination as compared to vehicle. Prasugrel monotherapy however, followed a similar pattern to DAPT producing greatly reduced expression of both markers. Significant reductions of PAC-1 expression were observed post therapy as compared to pre-treatment in all conditions tested and all

three inhibitor combinations were significantly reduced compared to vehicle. Prasugrel therapy in addition to NO and PGI_2 individually and in combination all reduced PAC-1 expression by 94%.

These results, using several methods of platelet testing indicate that even in the presence of effective $P2Y_{12}$ blockade, i.e. within consensus guidelines, the presence of NO and PGI_2 lead to very much higher levels of platelet inhibition. Similar patterns of inhibition were observed following DAPT and prasugrel therapy in aggregation, dense granule release and flow cytometry experiments. Whilst PGI_2+NO caused some inhibition in the presence of aspirin therapy, this was only in collagen induced aggregation and ATP release experiments. However, the effects on aspirin monotherapy were much less than those observed in the context of $P2Y_{12}$ receptor blockade where the potent three way synergy with PGI_2 and NO led to high levels of platelet inhibition in all methods tested.

Lastly, the effects of the various inhibitor combinations on the effector molecules of PGI_2 and NO, cAMP and cGMP, were tested. No significant differences in cGMP levels were found under any of the conditions tested. There were however, significant increases in levels of cAMP in both the aspirin plus prasugrel and aspirin plus ticagrelor groups following TRAP-6 and collagen stimulation. DAPT (aspirin plus prasugrel) with vehicle did not increase cAMP, nor did the addition of PGI_2+NO to pre-treatment PRP. However, the combination of all three significantly increased cAMP expression by 91% following collagen stimulation and 61% following TRAP-6 stimulation. I also noted a significant 91% increase in cAMP in the aspirin plus ticagrelor group treated with NO+PGI_2 following stimulation with collagen.

To conclude, this chapter highlights further the strong interactions between $P2Y_{12}$ receptor inhibition, PGI_2 and NO and the potential involvement of the cAMP and cGMP signalling systems. These results indicate that this synergy is mostly cAMP-related. The strong synergies between $P2Y_{12}$ inhibitors and the cAMP and cGMP signaling systems suggest strongly that the *in vivo* platelet reactivity in patients receiving DAPT will be a function of the level of $P2Y_{12}$ receptor blockade and the levels of endothelial-derived NO and PGI_2. This provides an explanation for different thrombotic outcomes in the presence of similar levels of platelet blockade; i.e. individual patients with different levels of endothelial function, or indeed disease-driven endothelial dysfunction, would have different levels of *in vivo* platelet inhibition for the same level of DAPT activity, as determined by *ex vivo* testing. This hypothesis will be further explored in patients with endothelial dysfunction in the next chapter.

Chapter 5: Endothelial function is a vital determinant of the therapeutic efficacy of $P2Y_{12}$ receptor antagonists in patients with peripheral arterial disease

5.1 Introduction

Having established in healthy volunteers that the endothelial mediators NO and PGI_2 influence platelet responses to $P2Y_{12}$ therapies, I sought to apply these findings to the clinical environment in patients with cardiovascular disease. I focused firstly on patients with a diagnosis of PAD, a common condition with a 12% prevalence in the general population [326] and 20-30% in the diabetic population [327]. Interestingly, despite dramatic improvements in revascularisation therapies there remains a very high morbidity and mortality associated with PAD with a mortality rate of up to 30% at 5 years [328].

There are several other reasons for choosing to study this particular patient group. Notably, both European and American PAD guidelines recommend a range of anti-platelet therapies for treatment which opens up the possibility of comparing and contrasting platelet responses to different therapies in this patient group. The ACCF/AHA guidelines give a 1A recommendation to the prescription of anti-platelet therapies to patients with symptomatic PAD [328]. More specifically, class 1B guidance is applied to both the use of aspirin (75mg-325mg) and clopidogrel (75mg) in these patients. The ESC guidelines specify a 1C recommendation for the use of anti-platelet agents in symptomatic PAD [329]. This is based on data from 9706 patients in 42 studies used in a meta-analysis by the antithrombotic trialists' collaboration which found a 23% reduction in vascular death, MI and stroke associated with the use of anti-platelet therapies in patients with peripheral arterial bypass, angioplasty or intermittent claudication [111]. Interestingly, low dose aspirin was found to be as efficacious as higher doses. The effectiveness of clopidogrel against aspirin was evaluated in a subgroup of 6452 patients with lower extremity arterial disease in the CAPRIE trial. At

follow-up at one year, there was a significant 23.8% reduction in events in those patients on clopidogrel as compared to aspirin with respective event rates of 3.9% and 4.9% [135]. The use of DAPT for the treatment of PAD however, is not indicated in this group as the small benefits associated with this therapy do not justify the increase in bleeding. One exception to this is patients with recent peripheral percutaneous revascularization, to the superior femoral artery or the iliac artery, for example, who may be prescribed DAPT for between 1 to 3 months as they may benefit from this intensified therapy [330]. However, definitive randomised data are lacking. Importantly, some patients with concomitant PAD and IHD will find themselves on DAPT following an ischaemic cardiac event or PCI.

Intriguingly, this latter patient group have a significantly worse prognosis following an ACS alongside other patient groups such as those with chronic kidney disease (CKD), DM and the elderly. This is reported in several observational studies but the reasons for this are not clear; it could be hypothesized that DAPT has reduced efficacy in this patient group. The PAMISCA study indicated PAD as a major predictor of adverse outcome in patients after ACS and that those patients with subclinical PAD also have an increased risk of cardiovascular mortality, MI, HF and angina compared to those without PAD [331]. They also suggest that the total atherosclerotic burden and more diffuse arterial damage in different arterial territories is associated with worse clinical outcomes. In a PAD subgroup analysis of the PLATO study at one year CV death, MI or stroke occurred in 19.3% of patients with PAD compared to 10.2% without PAD ($p<0.001$). The primary endpoint in those PAD patients treated with ticagrelor was 18% compared to those treated with clopidogrel 20.6%, which was not significantly different [332].

Thirdly, both PGI_2 and NO pathways are implicated in the pathogenesis of PAD. PAD patients have reduced endothelial function as a result of widespread PAD and associated disturbances in their endogenous production of NO and PGI_2 Reduced NO bioavailability has been demonstrated in patient with PAD, with diffuse vascular damage in different territories and elevated ADMA and SDMA levels predicting worse patient outcome [333] [331]. PGI_2 deficiency has also been implicated in the pathogenesis of PAD [68] [334]. This suggests that $P2Y_{12}$ receptor antagonist therapies may not be as effective in reaching their full therapeutic potential *in vivo* due to suboptimal endogenous inhibitor production and so reduce the three way synergy identified and discussed in previous chapters of this thesis. Moreover, in these patients the co-administration of aspirin with $P2Y_{12}$ receptor antagonists might be especially detrimental as it could potentially further reduce vascular PGI_2 production from already dysfunctional endothelial cells.

Taking into account the ideas elucidated above, it is most interesting to assess platelet reactivity in patients with PAD and to look at a range of individuals; treatment naïve patients, as well as those on the anti-platelet therapies, aspirin and clopidogrel monotherapy, and DAPT with aspirin plus clopidogrel. It is also of interest to define the patients' platelet function and responsiveness to endothelial mediators as compared to healthy volunteers.

5.2 Methods

5.2.1 Study participants

Male and female patients with stable PAD aged 18-85 years were recruited for participation in the study. Health status was determined though medical history and patient notes. Patients with PAD Fontain classification I-III were included in the study. Exclusion criteria included an acute cardiovascular event (< 4 weeks) and concomitant anticoagulation therapy.

5.2.2 Study protocol

Patients were recruited in the out-patient clinic where blood and urine samples were collected. Adherence to medication was assessed by interview during the visit.

5.2.3 Blood collection and preparation of PRP/PPP

Blood was obtained by venepuncture performed in the ante-cubital fossa using a 19 gauge butterfly needle into pre-filled vacutainers containing 3.2% tri-sodium citrate to a final ratio of anti-coagulant and blood of 1:9. PRP/PPP was prepared as described in section 2.2.4.

5.2.4 LTA

Baseline aggregation to AA (1 mM), ADP (5 and 20 µM), Horm collagen (0.4, 4 and 10 µg/ml), U46619 (10 µM) and TRAP-6 (25 µM) were determined, as described in section 2.2.7.1. Aggregations to TRAP-6 amide (25 µM) and Horm collagen (4 µg/ml) were measured after pre-incubation of PRP with vehicle (NaOH 0.01 M,) DEA/NONOate (100 nM) or PGI_2 (1 nM) individually or the combination of DEA/NONOate + PGI_2 for 1 minute at 37 °C.

5.2.5 Optimul

Platelet aggregation was assessed using the optimul method described in 2.2.7.3.

5.3 Results

5.3.1 PAD patient treatment groups

Blood and urine samples were collected from 42 patients with a proven diagnosis of stable PAD. Patients were prescribed a range of therapies (Table 5.1). In keeping with published guidelines, the majority of patients were prescribed monotherapy aspirin or clopidogrel and a smaller number were prescribed DAPT. A surprising proportion of patients did not take any regular anti-platelet therapy. Interestingly, two patients were prescribed cilostazol, a PDE inhibitor alongside DAPT. Cilostazol is highlighted in treatment guidelines as adjuvant therapy indicated in patients with lifestyle limiting intermittent claudication due to lower extremity PAD and receives a 1A recommendation to improve symptoms and increase walking distance [328, 329]. However, it is often poorly tolerated by patients due to side effects such as dizziness, headaches and diarrhoea. It is also contra-indicated in several patient groups such as those with congestive cardiac failure (CCF), severe tachyarrhythmias, recent MI or coronary intervention in the previous 6 months. Due to the low number of patients this data is not reported below. Baseline patient characteristics are described below (Table 5.2).

Therapy	Number of patients
Naïve	10
Aspirin	16
Clopidogrel	10
Aspirin + Clopidogrel	6

Table 5.1: Treatment groups of patients with PAD.

Characteristic		Number	Percentage
Median Age		64	
Male		36	86%
Smoker	Yes	24	57%
	Ex	7	17%
	No	11	26%
Ethnicity	Caucasian	25	60%
	Asian	13	31%
	Black	3	7%
Femoral angioplasty		6	14%
Myocardial infarction		6	14%
Diabetes Mellitus		20	48%
Hypertension		20	48%
Ischaemic heart disease		13	31%
Hypercholesterolaemia		13	31%
CABG		5	12%
Coronary stent		6	14%
Heart failure		4	10%
CVA		6	14%
Iliac stent		2	5%
Carotid artery stenosis		4	10%
COPD		11	26%
Chronic renal failure		1	2%
TIA		2	5%
AAA		1	2%
AF		1	2%

Table 5.2: Patients baseline characteristics

5.3.2 Characterisation of platelet responses to standard agonists by LTA

AA induced aggregation was significantly reduced in the aspirin therapy group compared to those patients that were not prescribed anti-platelet agents; 15±6 compared to 74±4% ($p<0.05$). Aggregation was further reduced in patients taking DAPT (1%±1%, $p<0.05$). Interestingly, aggregation was also significantly decreased by clopidogrel therapy (44±8%, $p<0.05$) (Figure 5.1.A). Aggregation following stimulation with ADP (5 µM) was not significantly reduced by aspirin therapy (naïve, 67±4%; aspirin, 49±6%). However, a significant reduction was seen in the clopidogrel and DAPT groups; 13±4% and 18±9%, respectively ($p<0.05$) (Figure 5.1.B). A similar pattern was observed with ADP (20 µM) whereby aggregation was significantly reduced by clopidogrel monotherapy and DAPT but not by aspirin therapy: naïve, 77±4%; aspirin, 64±5%; clopidogrel, 28±6% and DAPT, 21±8% (Figure 5.1.C). Collagen (0.4 µg/ml) induced 44±10% aggregation in naïve PRP. This was significantly reduced by clopidogrel (11±4%) but not by aspirin (23±7%) or DAPT (18±10%) (Figure 5.1.D). Following stimulation with collagen (4 µg/ml), aggregation was significantly decreased by DAPT (24±13% compared to 77±6% in naïve, $p<0.05$) but not by aspirin (56±7%) or clopidogrel (55±7%) (Figure 5.2.A). There were no significant differences in aggregation between treatment groups with collagen (10 µg/ml) (Figure 5.2.B). TRAP-6 induced aggregation in naïve PRP was 80±4%. This was not altered by aspirin or clopidogrel therapies (80±5% and 75±5%), but was reduced by DAPT (59±10%, $p<0.05$) (Figure 5.2.C). A similar pattern of aggregation was seen following stimulation with U46619 whereby aggregation was only significantly reduced in the DAPT group as compared to naive patients (57±8% compared to 80±4%). Neither clopidogrel (57±8%) nor aspirin significantly affected aggregation (79±4%) (Figure 5.2.D).

Interestingly, only aggregation induced by collagen (0.4 µg/ml) was significantly reduced in treatment naïve patients with PAD as compared to healthy volunteers. No significant differences were found between the two groups for any of the other agonists tested (Figures 5.1 and 5.2).

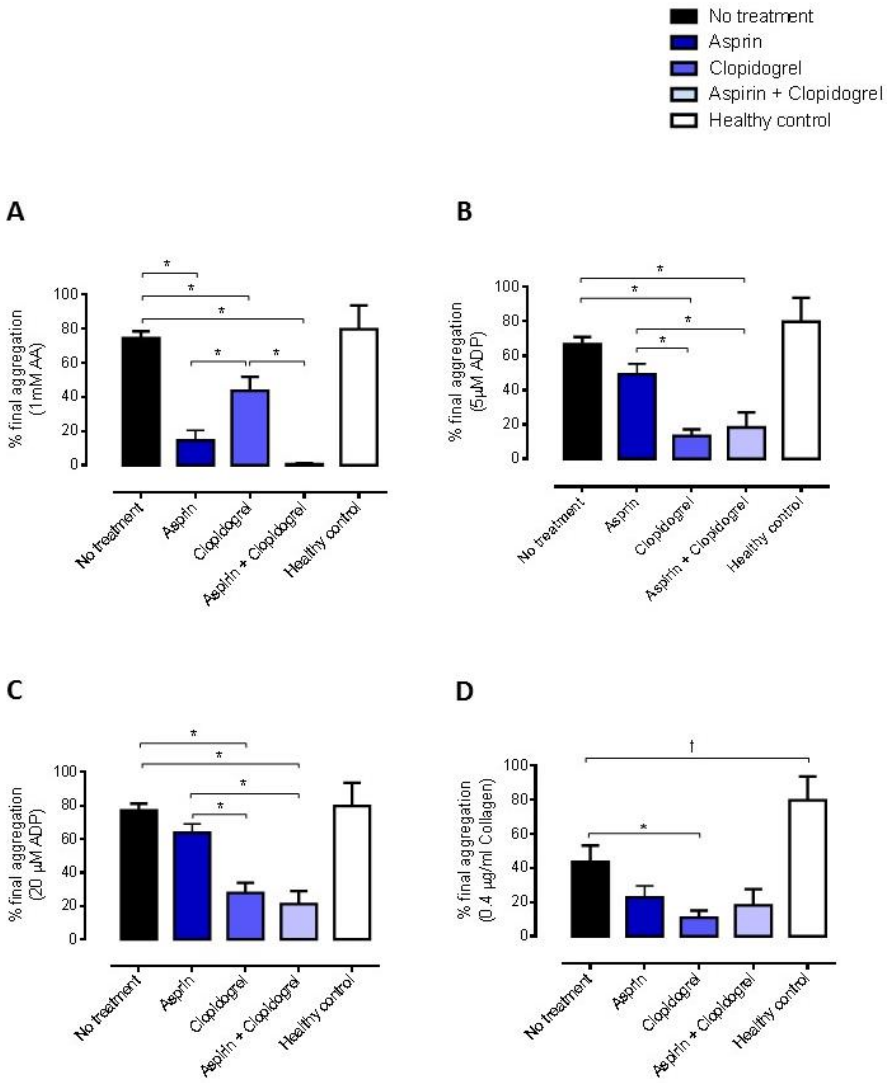


Figure 5.1: Standard agonist induced platelet aggregation in LTA for PAD patient subgroups. Standard LTA responses to A) AA (1 mM), B) ADP (5 µM), C) ADP (20 µM) and D) collagen (0.4 µg/mL) in patients with PAD and healthy volunteers. Data are presented as final aggregation (%, mean±SEM). Significance is shown as * $p<0.05$ between different PAD anti-platelet therapy groups and as † $p < 0.05$ between healthy volunteers and naïve PAD patients. N=50.

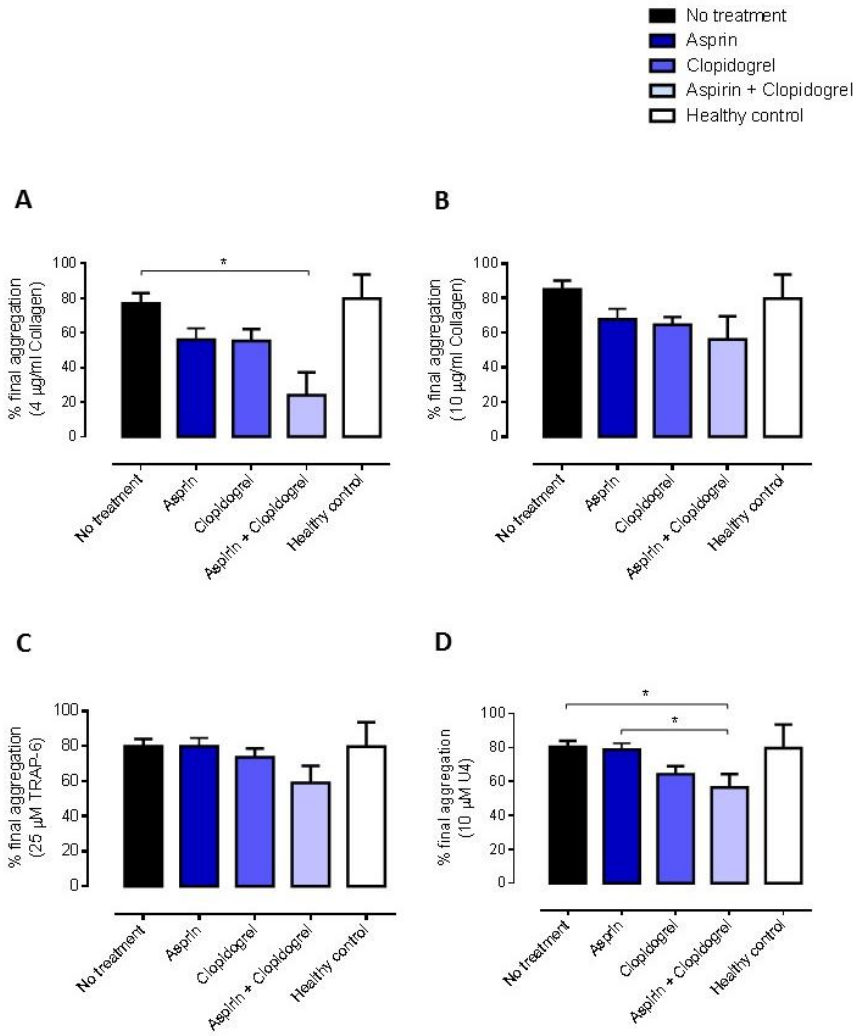


Figure 5.2: Standard agonist induced platelet aggregation in LTA for PAD patient subgroups. Standard LTA to A) collagen (4 µg/ml), B) collagen (10 µg/ml), C) TRAP-6 (25 µM) and D) U46619 (10 µM) in patients with PAD and healthy volunteers. Data are presented as final aggregation (%, mean±SEM). Significance is shown as * $p<0.05$ between different PAD anti-platelet therapy groups and as † $p < 0.05$ between healthy volunteers and naïve PAD patients. N=50.

5.3.3 Characterisation of optimul aggregation curves for patients with PAD on and off anti-platelet therapies

DAPT resulted in the lowest levels of platelet aggregation in response to all agonists tested. Clopidogrel also caused marked reductions in platelet responses to ADP, epinephrine, TRAP-6 and U46619, as did aspirin to platelet responses to AA, ristocetin and collagen (Figure 5.3).

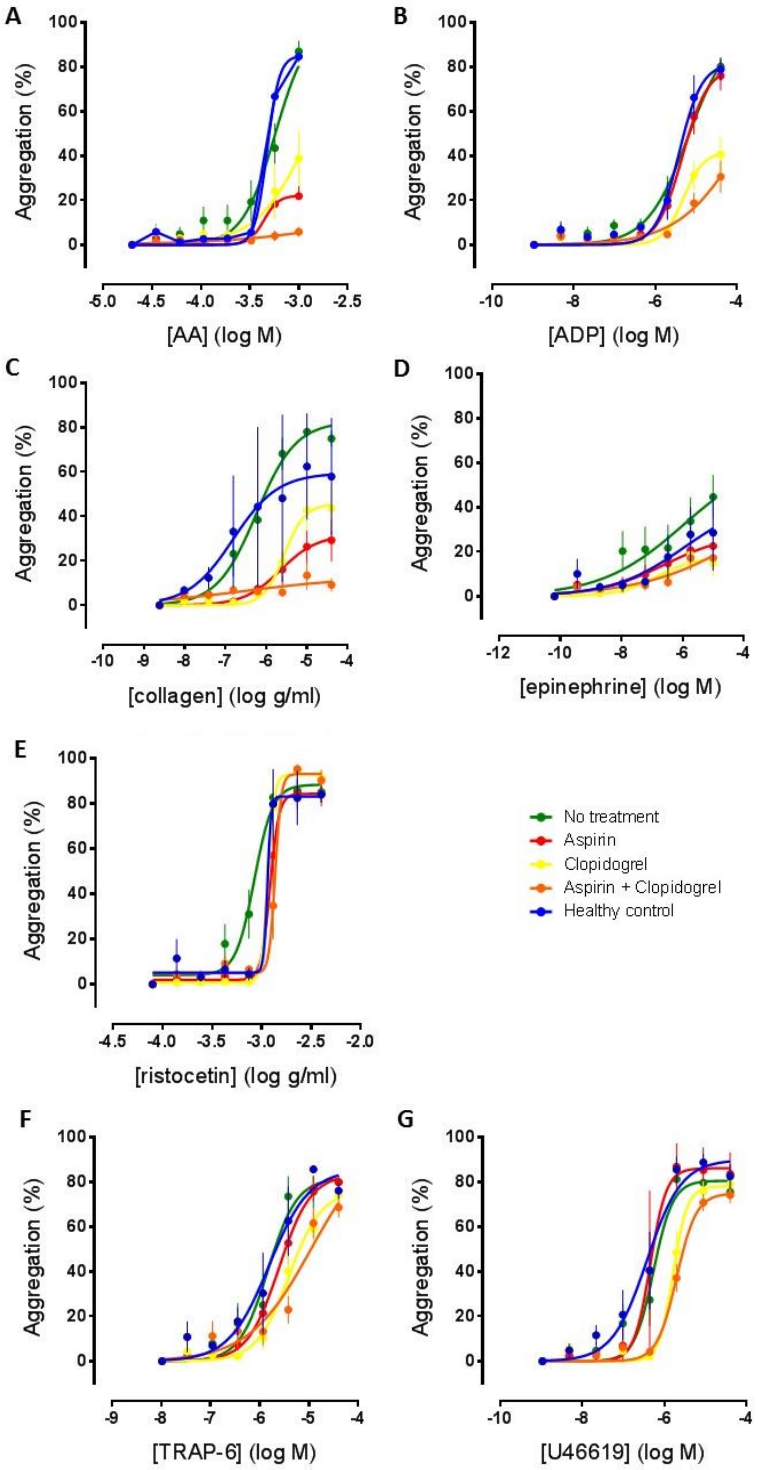


Figure 5.3: Standard optimul aggregation curves for PAD patients. Optimul plate aggregation traces for PAD patients; treatment naïve and those on standard anti-platelet therapies of aspirin (75 mg), clopidogrel (75 mg) and DAPT with aspirin and clopidogrel plotted against healthy controls. Agonists tested are shown A) AA, B) ADP, C) Collagen, D) Epinephrine, E) Risocetin, F) TRAP-6, F) U46619. Data are presented as final aggregation (%, mean±SEM). N=50.

5.3.4 The individual and combined effects of PGI_2 and NO following collagen stimulation

In treatment naïve patients, high levels of platelet aggregation were observed in response to collagen (4 μg/ml; 77±3%). This was not significantly reduced by the addition of PGI_2 or NO, 70±7% and 56±10%, respectively. There was a significant reduction however, following the addition of PGI_2+NO (35±10%, $p<0.05$) (Figure 5.4.A). Similar results were observed for the healthy control group (vehicle, 73±2% and PGI_2+NO, 56±6%, $p<0.05$) (Figure 5.4.E). In the aspirin treated group aggregation was significantly reduced by PGI_2 and PGI_2+NO (59±5% to 34±6% and 24±5% respectively, $p<0.05$) but not by NO (50±5%) (Figure 5.4.B). All three inhibitor combinations led to significant reductions in responses in clopidogrel treated patients (vehicle, 66±6%; NO, 46±7%; PGI_2 22±5% and PGI_2+NO, 9±2%, $p<0.05$) (Figure 5.4.C). DAPT with aspirin plus clopidogrel reduced aggregation to 29±15% in the presence of vehicle, with no further reduction following the addition of NO (29±15%). In the presence of PGI_2 and PGI_2+NO aggregations were 10±6% and 8±4%, respectively but these were not significantly different to vehicle (Figure 5.4.D).

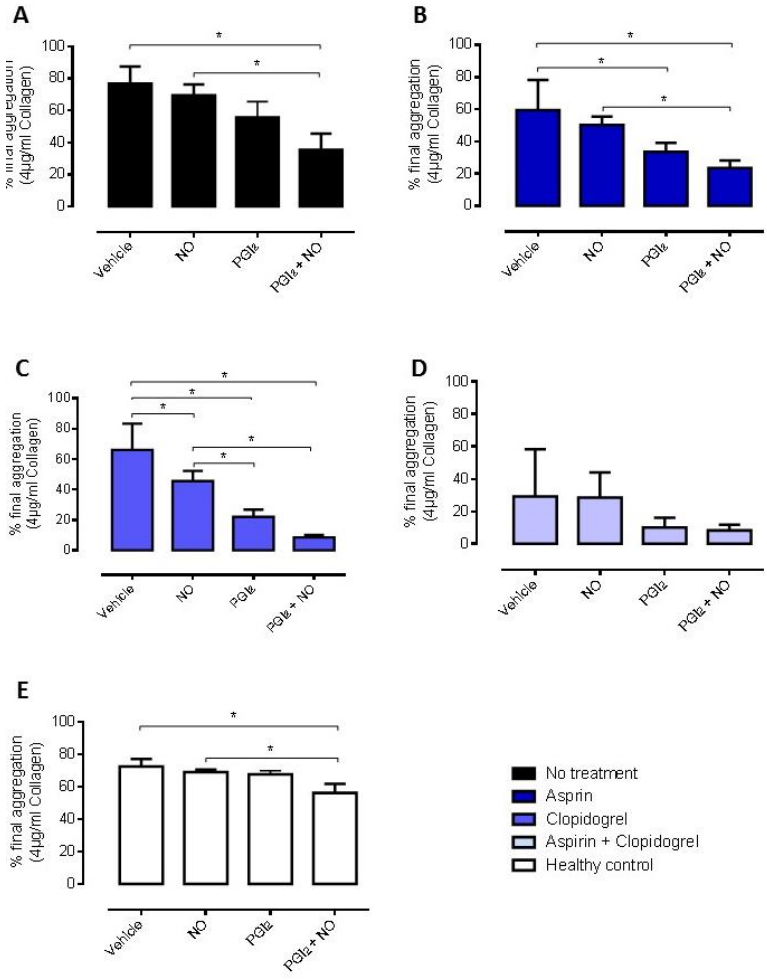


Figure 5.4: Interactions of NO and PGI_2 in anti-platelet treated and non-treated PAD patient groups and healthy controls: platelet aggregation following collagen stimulation. Bar graphs of platelet aggregation in response to collagen (4 µg/ml). Aggregometry was conducted in A) treatment naive PAD patients as well as those prescribed B) aspirin (75 mg), C) clopidogrel (75 mg), D) aspirin (75mg) plus clopidogrel (75 mg) and in E) healthy volunteer controls. Aggregometry was conducted in the presence of vehicle (NaOH, 0.01 M), NO (100 nM), PGI_2 (1 nM), or NO + PGI_2. Data are presented as final aggregation (%, mean±SEM). Significance is shown as * $p<0.05$. N=50.

Comparing the effects of respective endothelial inhibitors across different treatment groups (Figure 5.5), following collagen (4 µg/ml) stimulation high levels of platelet aggregation were observed in vehicle treated naive PAD patients (77±3%) and healthy volunteers (73±2%). Aggregation was significantly reduced by vehicle only in the DAPT treated patients (29±15%, p<0 05) compared to these. A similar pattern was observed in NO treated samples with only DAPT reducing aggregation (29±16, $p<0.05$) compared

to treatment naïve PAD patients and healthy volunteers (70±7% and 69±2%, respectively). Addition of PGI_2 led to significant reductions ($p<0.05$) in clopidogrel (22±6%) and DAPT (10±6%) treated groups whereas, aggregation remained high in naïve PAD patients (56±10%) and healthy volunteers (68±2%). Incubation with PGI_2+NO still led to high levels of aggregation in healthy volunteers (56±6%) but not in treatment naïve PAD patients (35±10%). PGI_2+NO significantly decreased aggregation in clopidogrel and DAPT patients; 8±2% and 8±4%, respectively. Importantly, aspirin therapy did not result in significant levels of platelet inhibition in any inhibitor condition tested (Figure 5.5).

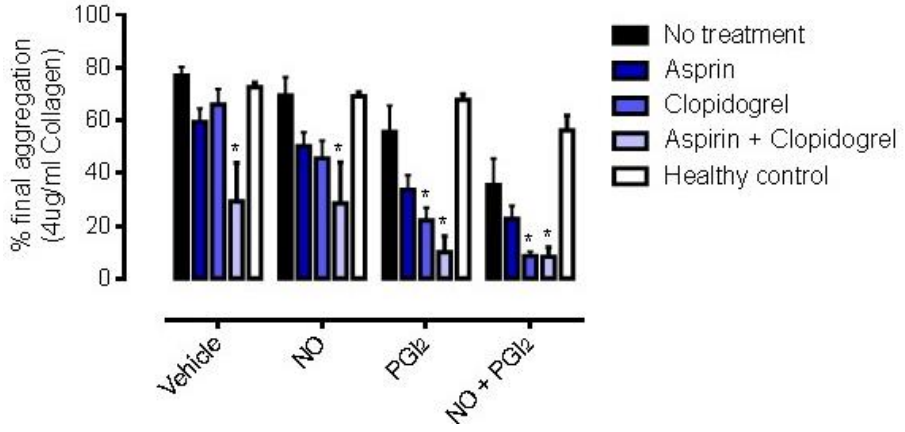


Figure 5.5: Effects of NO, PGI_2 and PGI_2+NO in PAD patient therapy sub-groups and healthy controls: platelet aggregation following collagen stimulation. Bar graphs of platelet aggregation in response to collagen (4 µg/ml). Aggregometry was conducted in treatment naive PAD patients, as well as those prescribed aspirin (75 mg) clopidogrel (75 mg), aspirin (75mg) plus clopidogrel (75 mg) and in healthy volunteer controls. Aggregometry was conducted in the presence of vehicle (NaOH, 0.01 M), NO (100 nM), PGI_2 (1 nM), or NO + PGI_2. Data are presented as final aggregation (%, mean±SEM). Significance is shown as * $p<0.05$ in inhibitor subgroups compared to naïve PAD patients. N=50.

5.3.5 The individual and combined effects of PGI_2 and NO following TRAP-6 stimulation

In samples from patients taking aspirin, addition of PGI_2 (73±5%) or NO (67±5%) alone did not inhibit platelet responses to TRAP-6 but the combination did (vehicle, 77±5 to 59±4%, $p<0.05$) (Figure 5.6.B).

In clopidogrel treated patients, aggregation was significantly reduced by the addition of PGI_2+NO (74±5% to 36±9%, $p<0.05$), but not by PGI_2 or NO alone (48±8% and 66±5%, respectively) (Figure 5.6.C). There were no significant differences observed for the DAPT patient group; vehicle, 59±10%; NO, 54±11%, PGI_2 34±12% and PGI_2+NO 31±12% (Figures 5.6.D). Neither did inhibitors reduce aggregation in PAD naïve or healthy volunteers compared to vehicle (Figures 5.6.A/E).

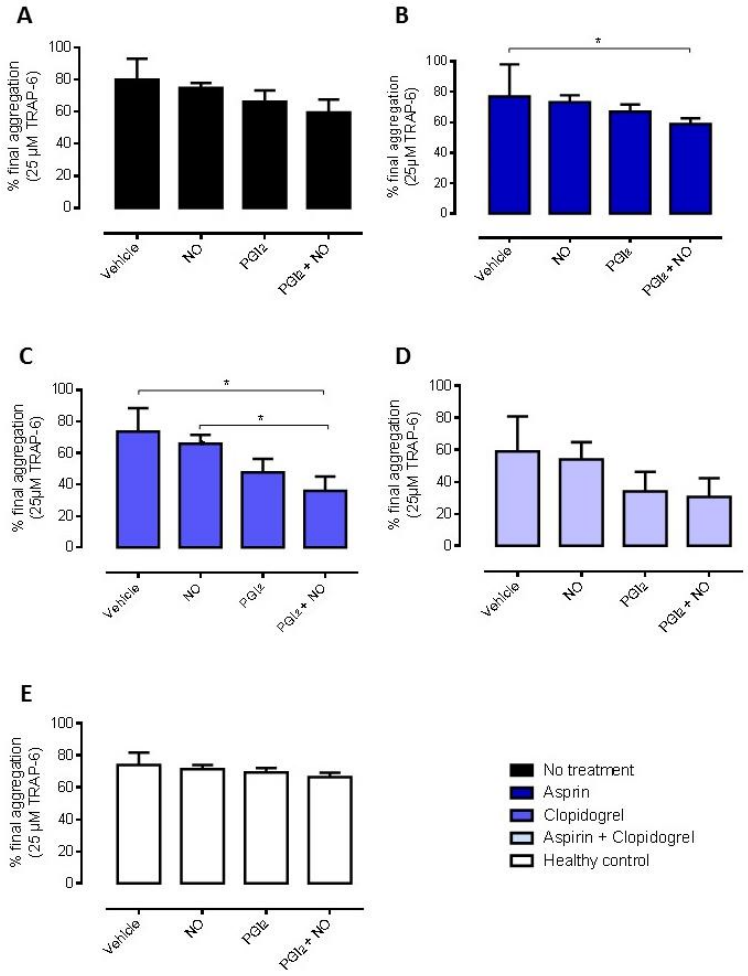


Figure 5.6: Interactions of NO and PGI_2 in treated and non-treated PAD patient groups and healthy controls: platelet aggregation following TRAP-6 (25 µM) stimulation. Bar graphs of platelet aggregation in response to TRAP-6 (25 µM). Aggregometry was conducted in A) treatment naive PAD patients, as well as those prescribed B) aspirin (75 mg), C) clopidogrel (75 mg), D) aspirin (75mg) plus clopidogrel (75 mg) and in E) healthy volunteer controls. Aggregometry was conducted in the presence of vehicle (NaOH, 0.01 M), NO (100 nM), PGI_2 (1 nM), or NO + PGI_2. Data are presented as final aggregation (%, mean±SEM). Significance is shown as * $p<0.05$. N=50.

On comparing the effects of corresponding endothelial inhibitors across different treatment groups following TRAP-6 (25 µM) stimulation (figure 5.7), I found no significant differences between those samples treated with vehicle or NO. PGI_2 treatment however, significantly reduced aggregation in DAPT patients (34±12%, $p<0.05$) compared to treatment naïve patients (67±5%) and healthy controls (69±3%). Aggregation remained high in samples from healthy controls and naïve PAD patients treated with NO+PGI_2; 66±6% and 67±3%, respectively. However, aggregation was significantly reduced ($p<0.05$) by both clopidogrel and DAPT with NO+PGI_2; 36±9% and 31±12%, respectively. Notably, again aspirin therapy did not reduce aggregation with any inhibitor combination tested.

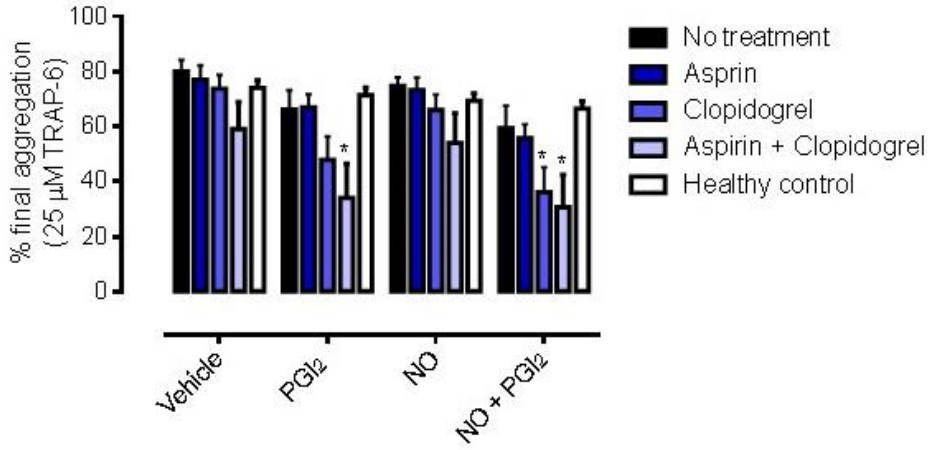


Figure 5.7: Effects of NO, PGI_2 and PGI_2+NO in PAD patient therapy sub-groups and healthy controls: platelet aggregation following TRAP-6 stimulation. Bar graphs of platelet aggregation in response to TRAP-6 (25 µM). Aggregometry was conducted in treatment naive PAD patients as well as those prescribed aspirin (75 mg), clopidogrel, (75 mg), aspirin (75mg) plus clopidogrel (75 mg) and in healthy volunteer controls. Aggregometry was conducted in the presence of vehicle (NaOH, 0.01 M), NO (100 nM), PGI_2 (1 nM), or NO + PGI_2. Data are presented as final aggregation (%, mean±SEM). Significance is shown as * $p<0.05$ in inhibitor subgroups compared to naïve PAD patients. N=50.

5.3.6 Comparison of platelet responses to standard agonists in PAD patients and healthy volunteers prescribed aspirin therapy

Lastly using LTA, platelet aggregation in PAD patients treated with aspirin was compared to that in healthy volunteers prescribed aspirin for 7 days. Interestingly, AA induced 15±6% aggregation in PAD patients compared to 2±1% in healthy volunteers. However, this did not reach statistical significance. Nor were any significant differences found between the two groups for other agonists tested (Figure 5.8).

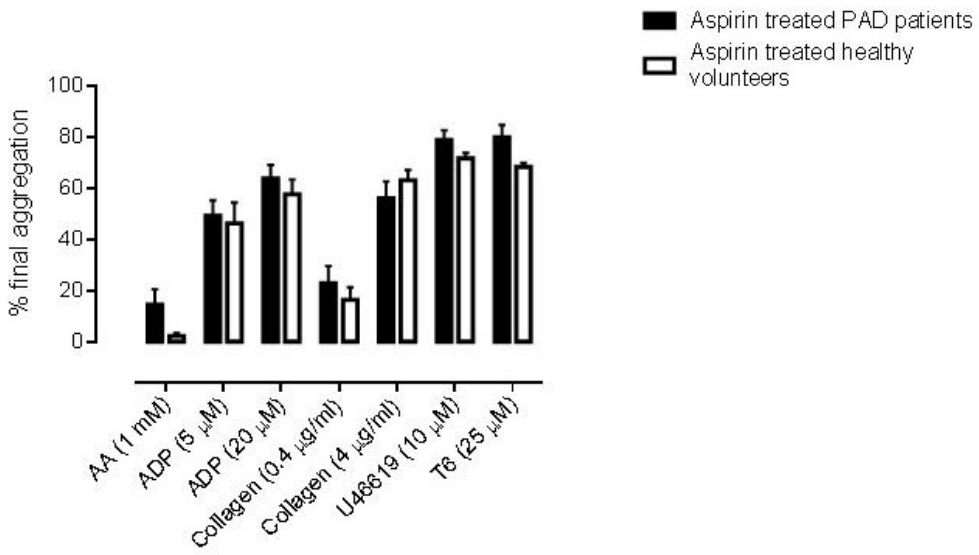


Figure 5.8: Standard LTA agonist induced platelet aggregation for patients with PAD and healthy volunteers prescribed aspirin therapy. Standard LTA responses to AA (1 mM), ADP (5 and 20 μM), collagen (0.4 and 4 μg/mL), U46619 (10 μM) and T6-amide (25 μM) in patients with PAD and healthy volunteers treated with aspirin therapy. Data are presented as final aggregation (%, mean±SEM). Significance is shown as * $p<0.05$. N=24.

5.3.7 The effects of PGI_2 and NO in PAD patients and healthy volunteers prescribed aspirin therapy

For both TRAP-6 (25 μM) and collagen (4 μg/ml), responses to PGI_2+DEA/NONOate were significantly different between the two treatment groups. Collagen induced aggregation was higher in the PAD patients (21±5%) compared to healthy volunteers (2±1%). TRAP-6 also induced higher levels of aggregation in the PAD aspirin treated patients as compared to aspirin-treated healthy volunteers (60±4% versus 28±9%) (Figure 5.9).

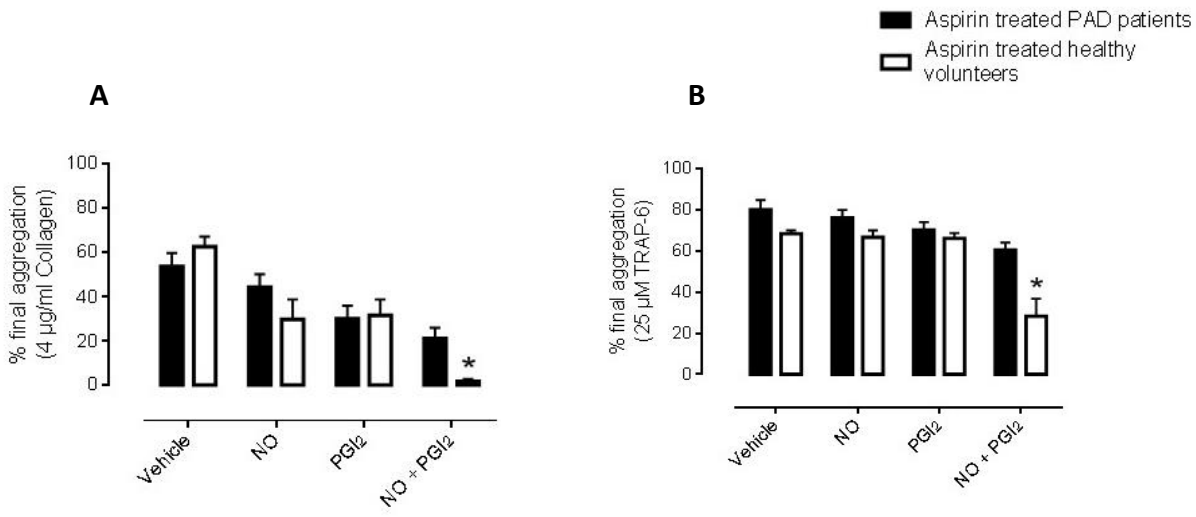


Figure 5.9: Responses to NO and PGI_2 in patients with PAD and healthy volunteers prescribed aspirin therapy. Bar graphs of platelet aggregation in response to A) collagen (4 µg/ml) and B) TRAP-6 (25 µM). Aggregometry was conducted in aspirin (75 mg) treated PAD patients, as well as healthy volunteers prescribed aspirin (75 mg). Aggregometry was conducted in the presence of vehicle (NaOH, 0.01 M), NO (100 nM), PGI_2 (1 nM), or NO + PGI_2. Data are presented as final aggregation (%, mean±SEM). Significance is shown as * $p<0.05$. N=24.

5.4 Discussion

In this chapter, I investigated platelet responses in patients with a diagnosis of PAD, a patient group associated with high morbidity and mortality and significantly worse outcome following ACS. I firstly sought to establish baseline reactivity in the 4 PAD patient groups (naïve, aspirin, clopidogrel and DAPT with aspirin plus clopidogrel) and compare these to healthy volunteer controls. In treatment naïve PAD patients I observed high levels of platelet aggregation (>70%) to all agonists tested other than collagen (0.4 μg/ml). Interestingly, collagen (0.4 μg/ml) induced aggregation was actually significantly reduced in PAD patients compared to healthy volunteers. There were however, no other significant differences found between FA values in these two groups, suggesting that despite being more prone to thrombosis *in vivo* the platelets of this patient group are not inherently more reactive than those from healthy volunteers. This supports the idea that it could be the *in vivo* environment which is vital in determining platelet reactivity and the presence or absence of the endothelial inhibitors, NO and PGI_2.

In aspirin treated PAD patients, AA induced aggregation was 15±6%, compared to 74±4% in non-treated PAD patients. A reduction in keeping with consensus statements of effective aspirin therapy (aspirin resistance previously defined as >20% aggregation when tested in LTA [335]). This level of aggregation was higher than that observed in healthy volunteers prescribed aspirin monotherapy for a week (2±1%), in chapter 4 of this thesis, but not significantly different. Together these data suggest that when tested *in vitro* aspirin, albeit a weak inhibitor of platelet activity, produces similar levels of platelet inhibition in PAD patients and healthy volunteers.

In those PAD patients taking clopidogrel, aggregation to ADP 5 µM (13±4%) and ADP 20 µM (28±6%), were in keeping with consensus statements of effective $P2Y_{12}$ therapy (clopidogrel resistance previously defined as >46% and >59% aggregation to ADP 5 µM and ADP 20 µM by LTA [336]). Interestingly, compared to data from the healthy volunteer studies, platelet aggregation in these patients was found to be significantly higher to only AA (44±8% versus 12±6%) and ADP 20 (28±6% versus 2±2%) as compared to those volunteers who had taken prasugrel therapy for one week. There were no differences in responses to collagen, TRAP-6 or U46619. PAD patients taking DAPT were also within consensus statements (ADP 5, 18±9% and ADP, 20 21±8%), although responses to ADP were significantly higher than those in healthy volunteers prescribed DAPT with aspirin plus prasugrel (ADP 5, 1±1%; ADP 20, 2±1%). No differences were seen for other agonists tested. However, direct comparisons between clopidogrel-treated PAD patients and prasugrel-treated healthy volunteers are difficult to make as prasugrel is a more powerful and reliable inhibitor of ADP.

I also investigated the effects of PGI_2 and NO individually and in combination on platelets from the PAD patients. Following TRAP-6 (25 µM) stimulation, the addition of PGI_2 and/or NO did not significantly affect platelet aggregation in PRP from treatment naïve PAD patients or those prescribed DAPT. Both clopidogrel and aspirin therapy significantly reduced platelet aggregation in the presence of PGI_2+NO compared to vehicle (77±5% to 59±4%, $p<0.05$). This reduction was greater in clopidogrel treated patients (74±5% to 36±9%, $p<0.05$) highlighting the additional synergy of $P2Y_{12}$ blockade beyond that of PGI_2 and NO alone. Interestingly, on comparing the effects of PGI_2+NO on different treatment groups to naïve patients (66±6%), clopidogrel and DAPT led to significant reductions in platelet aggregation (clopidogrel, 36±9%; aspirin + clopidogrel,

31±12%, p<0.05). Aspirin therapy alone however, did not (aspirin, 59±4%) further affect aggregation, providing additional evidence of the powerful three-way synergy between NO, PGI_2 and $P2Y_{12}$ receptor blockade.

I also investigated the effects of PGI_2 and/or NO on platelet aggregation in response to collagen (4 μg/ml). In healthy volunteers and treatment naïve PAD patients, NO or PGI_2 alone did not reduce aggregation however, the combination did (healthy volunteers; vehicle 73±2%, PGI_2+NO 56±6%, p<0.05: treatment naïve; 77±3%, PGI_2+NO 35±10%, p<0.05). PGI_2+NO (24±5%) also significantly reduced aggregation compared to vehicle (59±5%) following aspirin treatment however, not to the same extent as following clopidogrel treatment (vehicle, 66±6%; PGI_2+NO, 8±2%). In this latter case, aggregation was also reduced by NO (46±7%) and PGI_2 (22±5%) individually, explained by $P2Y_{12}$ blockade potentiating the effects of both NO and PGI_2 individually and more strongly together. DAPT led to similarly reduced aggregation with PGI_2+NO, 8±4%. When comparing the effects of different endothelial inhibitors on anti-platelet regimes, I found DAPT significantly reduced aggregation in all inhibitor conditions tested compared to samples from treatment naïve patients. Both clopidogrel and DAPT reduced aggregation in samples treated with PGI_2 (naïve, 56±10%; clopidogrel, 22±5% and DAPT, 10±6%) and with PGI_2+NO (naïve, 35±10%; clopidogrel, 8±2% and DAPT, 8±3%). Therefore, for both TRAP-6 and collagen stimulation significant interactions between PGI_2+NO and $P2Y_{12}$ blockade were noted with clopidogrel in both the absence or presence of aspirin. Notably, these interactions were not observed in samples taken from patients treated solely with aspirin. The synergistic relationship between PGI_2+NO and $P2Y_{12}$ blockade produced by far the highest levels of platelet inhibition in these samples.

In this chapter, I studied the effects of common anti-platelet therapies used at standard doses in PAD patients. This is important to note, as there is the potential during *in vitro* studies to employ drug concentrations that do not reflect that seen in clinical use, particularly when using pharmacokinetically short-lived drugs such as aspirin and clopidogrel. The data collected demonstrated that whilst aspirin and clopidogrel therapy significantly reduce aggregation induced by AA and ADP, respectively, high levels of platelet aggregation are still observed following stimulation with the primary agonists TRAP-6 and collagen. This is suggestive that *in vivo* these strong pro-thrombotic pathways would still be able to act and could lead to thrombosis despite apparently effective therapeutic intervention. However, similarly to what was seen in healthy volunteers, the endothelial mediators NO and PGI_2 strongly interact with $P2Y_{12}$ blockade to inhibit platelet activation, even that induced by primary agonists. The effectiveness of the anti-platelet therapies *in vivo* may therefore be strongly dependent upon PGI_2 and NO production.

Chapter 6: Endothelial function testing in healthy volunteers

6.1 Introduction

As outlined in section 1.11 there are several non-invasive, cost-effective and reproducible techniques that can be employed to assess endothelial function to help evaluate cardiovascular risk. Amongst these techniques is laser Doppler flowmetry (LDF), a technique which monitors skin microvascular blood flow. The skin response reflects responses taking place in other vascular beds and provides an assessment of the microvasculature [316]. Combining this technique with iontophoresis or post-occlusive hyperaemia (PORH) are effective ways of calculating endothelial function. Reactive hyperaemia (RH) involves the release of a pneumatic cuff following a period of occlusion produced by inflating the cuff to supra-systolic blood pressure and is believed to reflect NO and prostanoid availability. Iontophoresis is a technique describing the migration of charged substances through the skin by means of delivering a small continuous galvanic current to allow very small amounts of drugs to be administered non-invasively. The administration of acetylcholine (ACh) can be used to induce endothelial-dependent vasodilatation reflecting NO and prostaglandin participation and sodium nitroprusside (SNP) to test smooth muscle dependent, non-endothelial pathways of vasodilation [318]. Both these techniques are dependent on NO bioavailability although other factors such as prostaglandins and adenosine are also involved. Here, I have applied these methods to test endothelial function in healthy volunteers with a view to using an estimation of endothelial function as an adjuvant to *ex vivo* platelet function testing to better represent the net *in vivo* platelet function in patients.

6.2 Methods

6.2.1 Recruitment of healthy volunteers

Potential volunteers aged 18-40, both male and female underwent a health screen and gave verbal and written consent to participate. This process involved a detailed medical history, physical examination and observations including blood pressure, pulse rate and temperature. Those fulfilling the inclusion criteria with normal health profiles were recruited.

6.2.2 Study period

Microvascular perfusion and vasodilator responses were measured in a temperature-controlled room (22-25 °C) according to a standardised protocol on study days. Measurements were undertaken during the day (10:00–16:00), with participants asked to refrain from smoking and ingesting any food, tea or coffee for 2 hours prior to the experiment. Subjects were lying in the supine position with their forearm supported at heart level. Following a 20-min equilibration period, the forearm area was cleaned with alcohol and dried.

6.2.3 Iontophoresis

Two iontophoresis chambers were positioned over healthy skin (avoiding any area of lipodermatosclerosis, excessive hair follicles or superficial veins) 10 cm apart on the medial surface of the forearm and 5 cm from the antecubital fossa. 1% ACh and 1% SNP diluted in sodium chloride (NaCl) were injected into the anodal and cathodal iontophoresis chambers, respectively. Drug concentration and current were chosen to minimise nonspecific vasodilatory effects [337]. The laser Doppler probe was positioned through the centre of each chamber and LDF measurements were made using a DRT4

(Moor Instruments, UK) including skin temperature, flux and microvascular dose-response curves for each of the five iontophoretic challenges.

After achieving a stable recording of baseline flux, LDF responses to ACh and SNP were measured using a modified incremental-dose iontophoresis protocol, as previously described [338]. In brief, dose response curves for ACh and SNP induced vasodilation were characterised using the following procedure in order to apply incremental charge stimuli: 50 µA applied for 10 s (500 µCb), 75 µA for 10 s (750 µCb), 100 µA for 10 s (1000 µCb), 100 µA for 20 s (2000 µCb and 100 µA for 30 s (3000 µCb), with a 3 minute recording period between each dose. Measurements were repeated with the chambers at exactly the same site on a separate occasion to test for reproducibility of the technique.

6.2.4 PORH

Following a 10 minute recovery period, the chambers were repositioned and RH performed. A standard probe was mounted on the medial aspect of the right forearm 10 cm from the antecubital fossa. After a 120 second basal perfusion recording, 180 seconds of supra-systolic pressure occlusion (180 mmHg) of the forearm was followed by sudden release of the cuff and recording for 180 seconds. Resting perfusion level, peak hyperaemic response and duration of RH were measured.

6.2.5 Data analysis and statistical analysis

Data was obtained using a commercial software system (MoorVMS-PC Ver3.1, Moor Instruments, UK) before being transferred to a database (Excel 97) and analysed using statistical software GraphPad Prism v5.

6.3 Results

6.3.1 Responses to PORH and iontophoresis assessed by LDF

Endothelial function testing was conducted by two approaches, PORH and iontophoresis using LDF. For PORH, baseline and occlusion perfusion were easily recorded, as were the responses to occlusive hyperaemia. Figure 6.1 depicts a representative PORH chart for one of the healthy volunteer subjects tested while figure 6.2 and table 6.1 demonstrate the variables measured.

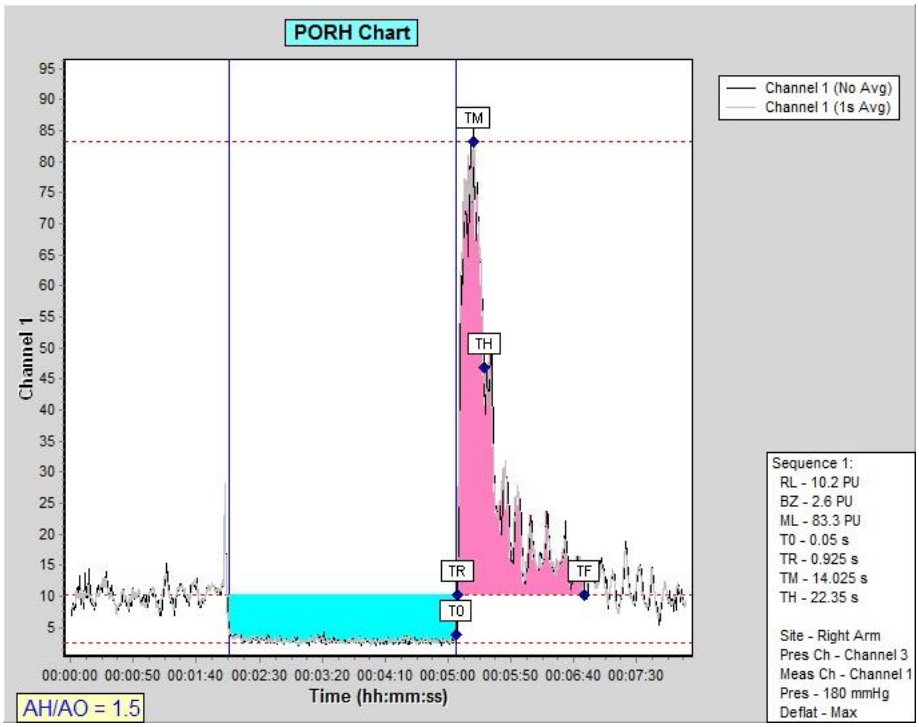


Figure 6.1: Representative PORH chart assessed by laser Doppler perfusion measurements of the forearm skin in a healthy volunteer. Channel 1 shows the resting level and biological zero at occlusion followed by the responses to occlusive hyperaemia including the maximal hyperaemic response following cuff release and the times to recovery, to maximal level, to half decay and to full decay.

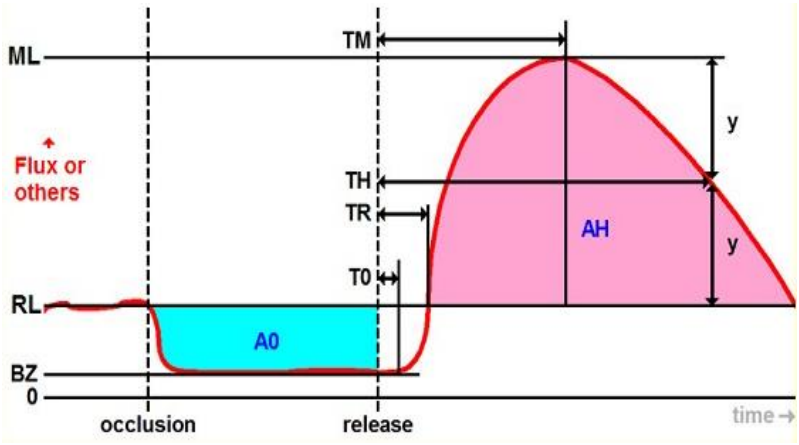


Figure 6.2: PORH - terminology and variables tested

Variable	Description
Resting level (RL)	The average level before inflation
Biological zero (BZ)	The average value during the second half of pressure holding
Maximum level (ML)	Maximum value after release of cuff pressure
Time to recovery (TR)	Interval between trace and restoring to RL
Time to maximal level (TM)	Interval between release and maximum value
Time to half decay (TH)	Interval from release to mid-decay value after maximum

Table 6.1: PORH - terminology and variables tested

Figure 6.3 highlights a representative trace obtained following iontophoresis of both ACh and SNP. The currents applied were imperceptible to most subjects while a slight tingling sensation was described by some. Compared to basal skin perfusion, graded iontophoretic administration of ACh and SNP resulted in successive perfusion increases.

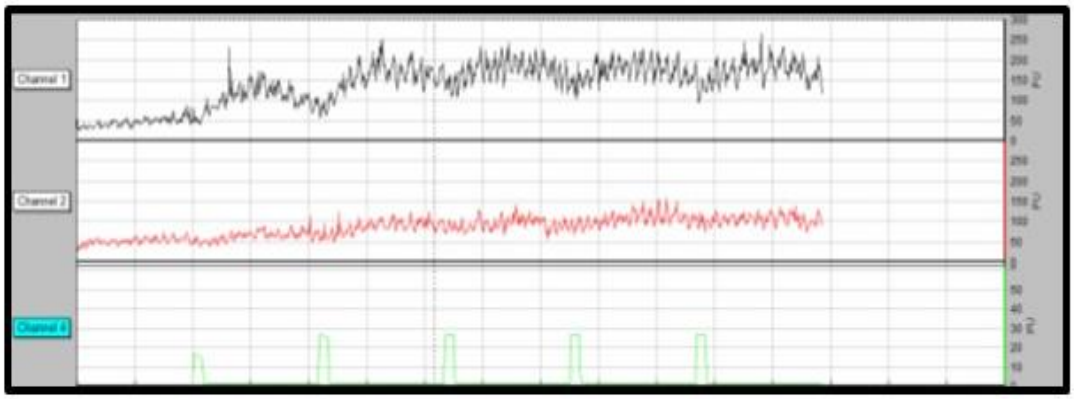


Figure 6.3: Representative trace of iontophoretic responses following administration of ACh and SNP assessed by laser Doppler perfusion measurements of the forearm skin in a healthy volunteer. Channel 1 represents iontophoresis of ACh and channel 2 administration of SNP with current bursts also displayed. Periods of iontophoresis are indicated: 0.50 mC (50 µA for 10 s), 0.75 mC (75 µA for 10 s), 1 mC (100 µA for 10 s), 2mC (100 µA for 20 s) and 3mC (100 µA for 30 s).

6.3.2 PORH testing by LDF in healthy volunteers

To be able to conduct endothelial function testing in future healthy volunteer platelet studies and in patients with cardiovascular disease the methodology was first tested in healthy controls to establish reproducibility. This was done in 10 healthy volunteers where the maximum value after release of cuff pressure (ML) response was recorded and repeated to give a total of two recordings per individual. The mean of the two peak hyperaemic recordings in these individual volunteers are displayed in perfusion units (PU) in figure 6.4 to demonstrate their normal distribution. The minimum mean ML recorded was 33 PU and the highest 129 PU. Mean ML+SEM for the group of 10 volunteers was 75±8 PU. Mean resting perfusion (RL) was 13±1 PU.

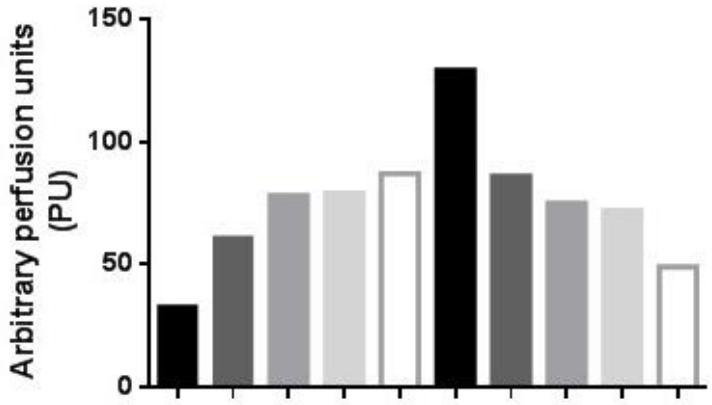


Figure 6.4: Distribution of mean peak hyperaemic values for the 10 healthy volunteers tested

Peak hyperaemic responses were repeated a total of two times to test the reproducibility of the technique. Repeated recordings are shown in the graphs in figure 6.5 below. The mean % difference between the two ML for the 10 individuals was 11±3%. The 25th percentile, median, 75th percentile and maximum % differences were 5, 10, 17 and 30%, respectively. Results for % difference per individual healthy volunteer are tabulated in Table 6.2 below.

ML 1	ML 2	% difference
72	86	17
45	52	14
76	81	6
88	84	5
64	87	30
73	72	1
36	30	18
93	80	15
62	59	5
127	131	3

Table 6.2: PORH - maximum values. ML 1 recorded on visit one and ML 2 recorded on visit two in each health volunteer. % difference between values ML 1 and ML 2.

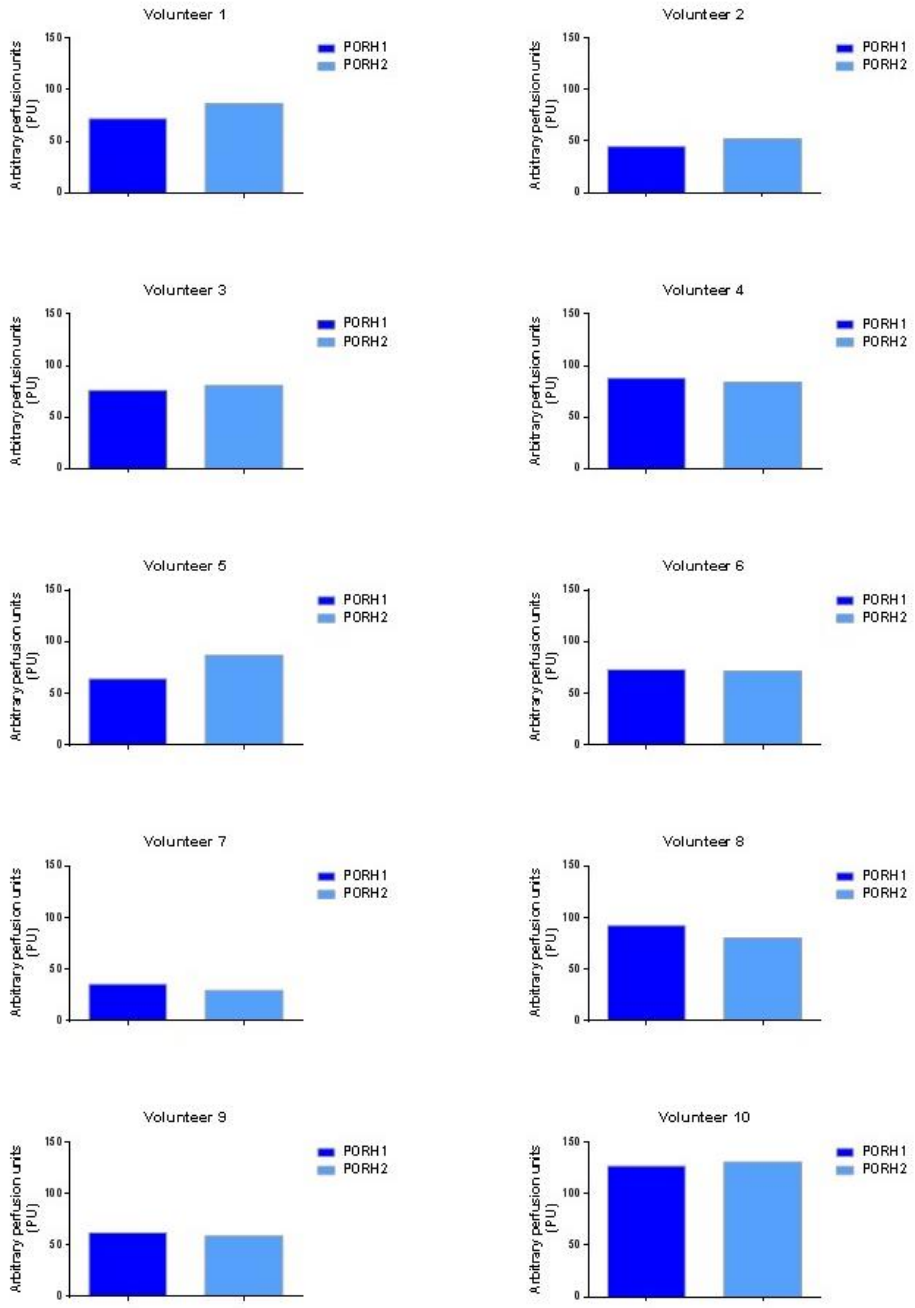


Figure 6.5: Individual maximum hyperaemic responses in healthy volunteers. ML recorded by LDF on two separate occasions to demonstrate reproducibility of the technique in healthy volunteers.

6.3.3 Forearm skin perfusion responses following graded iontophoresis of ACh and SNP determined by laser Doppler perfusion measurements in healthy volunteers

In 6 healthy volunteers endothelial function was assessed by iontophoresis of ACh (1%) and SNP (1%) through the skin using the following periods of iontophoresis: 0.50 mC (50 µA for 10 s), 0.75 mC (75 µA for 10 s), 1 mC (100 µA for 10 s), 2 mC 4, (100 µA for 20 s)

and 3 mC (100 µA for 30 s). Basal skin perfusion and successive increases in perfusion were recorded following this graded iontophoretic administration of ACh and SNP. Experiments were repeated twice in each volunteer. The mean basal skin perfusion response to SNP in all volunteers was 14±2 PU. This increased to 21±6, 32±8, 38±10, 66±12 and 84±10 PU with each application of SNP. For ACh, mean basal skin perfusion was 15±2 PU. With each application of ACh this increased to 23±4, 34±5, 45±5, 66±8 and 85±10 PU. Figure 6.6 represents the ACh and SNP traces obtained in my subject group.

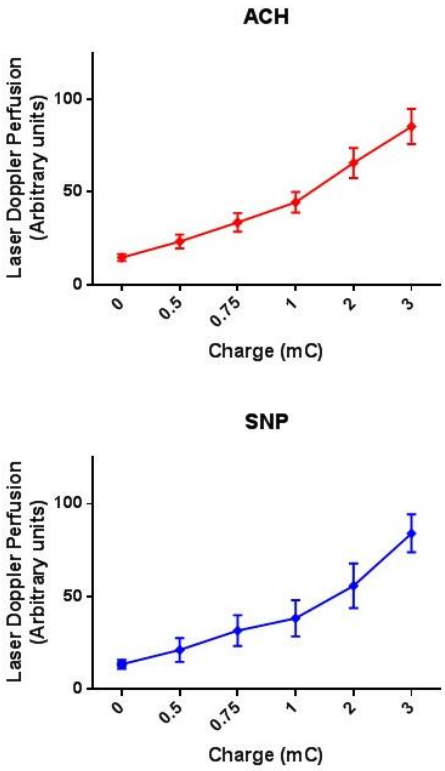


Figure 6.6: Laser Doppler perfusion measurements following graded iontophoresis of ACh (above) and SNP (below) in healthy controls. Data are presented as laser Doppler perfusion (Arbitrary units, mean±SEM). N=6.. Iontophoresis periods: 0.50 mC (50 µA for 10 s), 0.75 mC (75 µA for 10 s), 1 mC (100 µA for 10 s), 2 mC 4, (100 µA for 20 s) and 3 mC (100 µA for 30 s)

When represented as % change from baseline, SNP led to a 45±15, 126±24, 177±35, 349±58 and 645±69% increase in perfusion. ACh increased skin perfusion 66±14,

137±26, 258±46, 443±81 and 640±123% compared with basal levels. Detailed results are shown in figure 6.7 as % increase from baseline for each individual healthy volunteer.

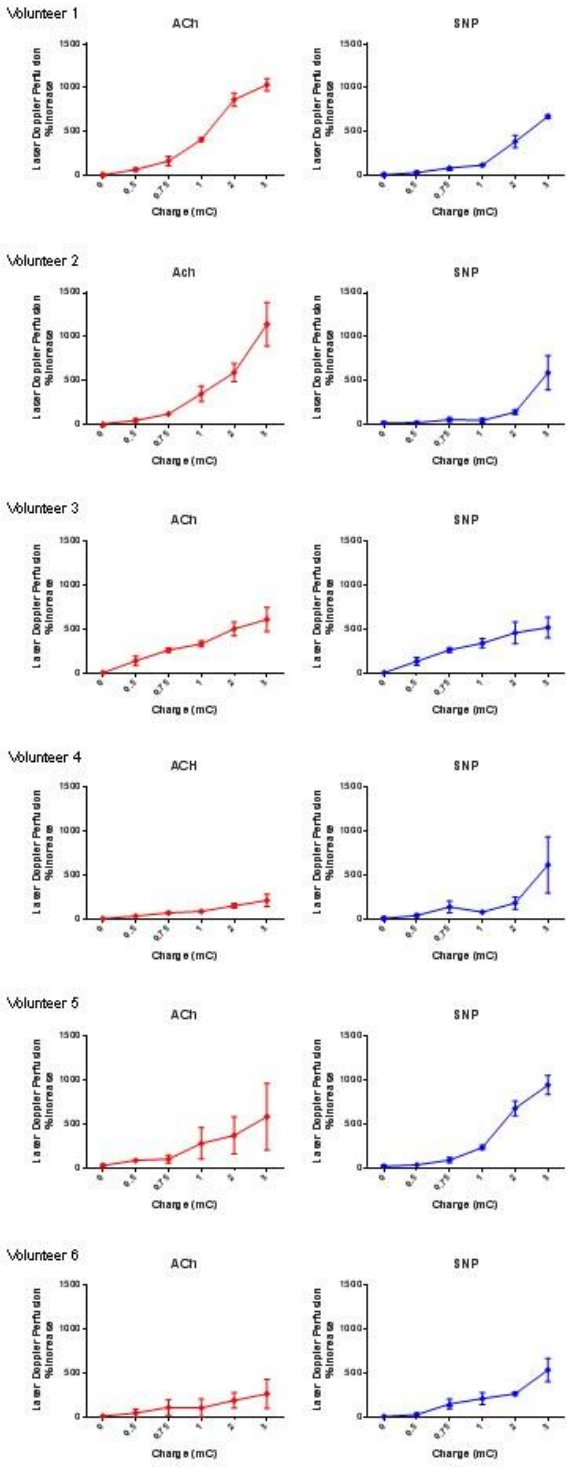


Figure 6.7: Laser Doppler perfusion measurements following graded iontophoresis of ACh and SNP displayed as % increase from baseline

6.4 Discussion

In this chapter I conducted pilot two tests of approaches to measure endothelium-dependent and endothelium-independent vasodilation in healthy volunteer subjects. The approaches used were laser Doppler techniques coupled with iontophoresis and PORH. These are recognised to be reliable methods of testing endothelial function and have previously been applied in clinical studies across a range of patient groups including those with hypertension [339], heart transplants [340], diabetes and venous insufficiency [338], and coronary artery disease, as well as healthy volunteer cohorts [341]. However, these approaches have not been previously used an adjuvant to assessing platelet reactivity.

The PORH studies were well tolerated by study subjects and baseline, occlusion and post-occlusive responses were easily recorded. PORHmax (ML) was chosen as my study variable as it has previously been shown to be a reproducible parameter for measuring microvascular reactivity during RH. In fact, in some studies it has been demonstrated to be the most reproducible index [342]. Although not fully standardised, PORH protocols are less variable than other methods used to test endothelial function. Most studies will use a pneumatic cuff inflated to a supra-systolic pressure (between 20 mmHg above systolic and 300 mmHg) with an occlusion time of 3 to 5 minutes. The studies reported here were based on a previous study establishing PORH using laser Doppler perfusion of the forearm as a sensitive and valid method to measure microvascular function. Keymel *et al* found that 3 minutes of forearm occlusion was sufficient to induce maximal vasodilation during PORH as indicated by maximal increase in perfusion unit (PU) amplitude that did not further increase after a total of five-minute occlusion [341]. In

these experiments it was therefore, decided to inflate the cuff to 180 mmHg for 3 minutes.

Using this method the PORHmax responses were found to follow a normal distribution, as one might expect. The mean PORHmax response was found to be 75±8 PU which is in keeping with previous studies in healthy controls reporting a population mean of 88±17 PU relative increase in perfusion post 180 second occlusion [341]. Reproducibility was high with a mean intra-individual variability of 11±3% and a range of 1-30%. This is comparable to previous studies reporting intra-individual variabilities of 12-21% [341]. I observed a wide range in inter-individual values of 33 to 129 PU which is higher than perhaps might be expected in healthy volunteers, previous studies have reported inter-individual variance rates of 14-26%. However, the present study cohort included males and females of both Asian and Caucasian race, aged between 23 and 38 with different life style factors and therefore, some variability in endothelial function could be expected. Some day-to-day and diurnal variation can also be expected which was more difficult to control in this pilot study although I did attempt to reduce diurnal and positional variability as best as possible.

Iontophoresis coupled with LDF allows for assessment of real-time changes of skin blood flow after the administration of different vasoactive substances without systemic effects. In recent years, iontophoresis drug delivery accompanied by laser Doppler technology has been increasingly used to measure endothelial function. Importantly, Hansell *et al* found a significant correlation between vascular responses to endothelial-dependent vasodilatation in the skin microvasculature and large arteries (brachial artery), using LDF and FMD, respectively [343]. My technique was well tolerated by

subjects with a tingling sensation perceived by some. One volunteer did suffer a very mild but completely reversible local allergic reaction to SNP and skin irritations have previously been reported in some individuals [344]. As is good practice, I carried out mild epidermal stripping on the iontophoresis area to clear away any surface lipids or keratinocytes in order to subsequently lower skin resistance and facilitate drug delivery.

As previously mentioned, ACh introduced into the skin by iontophoresis causes endothelium-dependent vasodilation, which can be compared with the endothelium independent vasodilator effect of SNP, a NO donor. Unfortunately, iontophoresis protocols are not standardised. Protocols from different laboratories vary widely in design and most are complicated and time consuming. There is a need for a simple, reproducible method but as this is not established I optimised the protocol using previously published literature. After achieving a stable recording of baseline flux, LDF responses to ACh and SNP were measured using a modified incremental-dose iontophoresis protocol, as previously described by Klonizakis *et al* in both healthy volunteer and patient studies [338, 345]. Most groups prefer to obtain a dose response curve by changing the duration of a constant current (e.g., 100 μA) or by a gradual increase in current over time (10-100 μA). The latter has the advantage that it looks at the vascular response to an agonist at low currents minimising nonspecific, current-induced galvanic responses [346].

Drug concentrations and currents were chosen so as to minimize nonspecific vasodilatory effects, in particular reducing galvanic effects which are thought to occur due to current induced release of neuropeptides (e.g., substance P, bradykinin, calcitonin gene-related peptide) [337]. A range of vehicles have been used to dilute

drugs for iontophoresis including deionized water, tap water, and NaCl. These vehicles have at times been found to induce dermal vasodilation, referred to as a "galvanic response" [346]. This so called 'galvanic response' leads to local vasodilation, initiated by C-fibre activation which can be due to differing pH changes caused by electrolysis of water and/or acidosis sensed at the anode and alkalosis at the cathode [347]. I used NaCl as the iontophoresis vehicle solution as it has been shown to have a negligible vasodilator effect [348] and I observed acceptable day to day reproducibility using this. Whilst reproducibility was acceptable, it could have been higher but laser Doppler coupled with iontophoresis has in fact, been associated with poor reproducibility previously and mean day to day variances in ACh response between 6.4%-42% have been reported. This is thought to be explained by the spatial heterogeneity of skin blood flow and movement artefacts. Drug delivery is also influenced by skin resistance, which varies significantly between individuals and across different skin areas. Ramsay *et al* found that higher skin resistance is associated with smaller vasodilator responses for both SNP and ACh [344].

Traditional data analysis methods measure the magnitude of the change in flux. Maximum perfusion is affected by the duration of endothelial and smooth muscle cell exposure to specific stimuli and is affected by the duration and the value of electrical current. Similar basal perfusion rates were observed for both ACh and SNP, 15±2 and 14±2 PU, respectively. The 5 applications of ACh and SNP each led to increased skin perfusion with the final level of skin perfusion obtained by each drug being similar, 85±10 PU for ACh and 84±10 PU for SNP. The iontophoresis of ACh elicited significant increases ($p<0.05$) in perfusion at every stage of iontophoresis. SNP elicited a significant increase from the third period of iontophoresis onwards (1 mC to 3 mC). Represented

as % increase from baseline, ACh induced skin perfusion at the end of the iontophoresis period was increased 640±123%. A similar increase in perfusion following SNP iontophoresis was observed, with levels increasing 645±69% in my heathy volunteers. This value of maximum vasodilation is similar to values of 700-800% change obtained in healthy control groups in other published studies. The matching values of ACh and SNP maximal perfusion is suggestive of similar endothelial and non-endothelial dependent reactions in my healthy controls, as would be expected in those without endothelial dysfunction. Generally, the relative contributions of endothelial factors to these vasodilator responses is not well understood with some suggesting that prostanoid-dependent mechanisms do not significantly contribute to endothelium-dependent vasodilation [349] and others suggesting that cutaneous vasodilatation is mainly driven by a prostanoid-dependent mechanism [350]. SNP, on the contrary does not stimulate endogenous NO production but rather reacts with tissue sulfhydryl groups under physiologic conditions to produce NO directly and thereby stimulate SMC relaxation [346]. SNP is therefore, used as an endothelium dependent control. A reduction in vascular response in ACh with no concurrent reduction in SNP response is indicative of endothelial dysfunction. A reduction in SNP response can be interpreted as a structural change within the vessel or as a reduction in NO activity or availability caused by, for example, high oxidative stress.

My future work will use laser Doppler combined with iontophoresis and/or PORH to assess microvascular function in patients at risk of and with established cardiovascular disease and in healthy volunteers pre- and post-intervention with agents such as naproxen, aspirin and $P2Y_{12}$ receptor antagonists that affect both endothelial and platelet function. This technique is a particularly attractive approach to try to

understand the net influences on platelet and endothelial function, as the microcirculation may well be the initial site of endothelial damage in subjects at risk of cardiovascular disease [351].

Chapter 7: Platelets influence clot microstructure as assessed by fractal analysis of viscoelastic properties

7.1 Introduction

As described in chapter 1, there exists a complex interplay of platelets and coagulation in atherothrombosis. Platelets are critical to the processes of atherothrombosis, driving thrombus formation through a cascade of complex intracellular signalling pathways and powerful positive feedback loops [95]. Platelets also have a central role in the cell-based regulation of the coagulation system [352-354]. Because of the fundamental involvement of platelets in atherothrombosis, anti-platelet drugs that target amplifying secondary platelet agonists are the standard of care for the prevention of thrombotic events in patients with cardiovascular disease.

Coagulation pathway changes also play a central role in clot propagation, a process that is affected by many factors both genetic and environmental [355]. Configuration of the fibrin network has been shown to be a vital determinant of clot stability and susceptibility to fibrinolysis [356, 357] with clot permeability being the rate limiting factor for the activity of plasmin, the fibrin network degradation enzyme. Clots composed of compact thin fibres are associated with thrombotic events [358, 359] and clot microstructure appears unfavourably altered in CAD and other conditions associated with thrombosis [360-363] and is also associated with adverse events following PCI. Despite the interwoven nature of platelet activation and the coagulation system in thrombosis however, few studies relate both analysis of protein and cellular parts of coagulation in the same population.

The following chapter investigates the complex interplay of platelets and coagulation pathways in clot microstructure. This is of relevance as it has been suggested that there

is a definitive diagnostic potential in characterising clot microstructure and modulation of clot architecture as a possible treatment for thrombosis [364]. However, many techniques that assess the mechanics and quality of clot architecture rely on processed samples using altered blood in remote laboratories, limiting their clinical use.

Viscoelastic properties have been shown to be sensitive measures of fibrin polymerisation and blood clot structure [365] and these properties can be quantified using small amplitude oscillatory shear rheometry to measure gel point (GP) [366]. GP is a multi-faceted biomarker that defines the transition between viscoelastic fluid and viscoelastic solid states during gelation and in coagulating blood GP marks the establishment of the incipient clot. GP provides three related biomarkers all calculated from the one measurement; time taken to reach GP or gel time (T_{GP}), clot strength (G'_{GP}) and the fractal dimension (d_f) of the incipient clot, which provide a quantitative assessment of clot microstructure. Structure of the early clot, as quantified by d_f has been established as providing a template for the mature clot when analysed based on viscoelastic and imaging data and quantified by fractal analysis [367], a technique used in biology and medicine to characterize nonlinear growth in branching network structures Reduced d_f is associated with more permeable, less branched and mechanically weaker clots and a raised d_f corresponds to a tightly packed, highly branched clot that is stronger and less permeable. The value of T_{GP} and d_f as new biomarkers of haemostasis has been highlighted in several recent studies [368-371]. T_{GP} and d_f are used as parameters to investigate clot architecture in this chapter. Clot microstructure is further investigated by projecting random fractal aggregates (RFAs) as a function of d_f, representative of ensuing clot development which I then related to platelet reactivity. To produce these assessments of platelet function, standard tests of

aggregation and activation were used to determine the effects of individual anti-platelet therapies on platelets taken from healthy volunteers treated with standard anti-platelet therapies. The influences of these therapies on clot structure were then determined using advanced rheological analyses of incipient fibrin clots. Together these approaches provided an insight into the complex interplay of platelets in the formation of clot microstructure.

7.2 Methods

7.2.1 Study participants

16 healthy, male volunteers (aged 18-40 years) participated in an initial single anti-platelet therapy study. Health status was determined though medical history and physical examination, including blood pressure, pulse rate, blood chemistry and urinalysis. Volunteers with normal clinical profiles were included in the study. Subsequently, a further 20 healthy volunteers were recruited in an identical manner for a follow-up DAPT study.

7.2.2 Study protocol

All healthy volunteers abstained from aspirin, NSAIDs and any other anti-platelet therapy for 14 days before commencing the studies. Two groups of 8 volunteers received monotherapy aspirin (75 mg) or prasugrel (10 mg) for 7 days. Subsequently, two groups of 10 volunteers received DAPT with either aspirin (75 mg) plus prasugrel (10 mg) or aspirin (75 mg) plus ticagrelor (90 mg) for 7 days. Compliance was assessed by interview. Blood samples were collected prior to and following drug treatment in both studies on day 0 and day 7.

7.2.3 Blood collection

Blood was obtained by venepuncture performed in the ante-cubital fossa using a 19 gauge butterfly needle.

7.2.4 Laboratory markers

Blood was taken for full blood count (FBC) analysis, including platelet count and haemoglobin into 4ml BD EDTA vacutainers and into 4.5ml BD citrate vacutainers for routine coagulation studies, including prothrombin time (PT), activated partial

thromboplastin time (APTT), activated partial thromboplastin time ratio (APTTr), INR and fibrinogen. Measurements were made by use of automated analysers, Sysmex XN-2000 and Sysmex CS-2100i.

7.2.5 Platelet function

Blood for platelet studies was collected into tri-sodium citrate. PRP/PPP was prepared, as described in section 2.2.4.

7.2.5.1 LTA

Baseline aggregation to AA (1 mM), ADP (5 and 20 µM), Horm collagen (0.4, 4 and 10 µg/ml), U46619 (10 µM) and TRAP-6 (25 µM) were determined, as described in section 2.2.7.1.

7.2.5.2 ADP + ATP secretion

ADP + ATP secretion of PRP was evaluated by luminescence in the presence of Chrono-Lume reagent after stimulation with collagen (4 µg/mL), as described in section 2.2.8.

7.2.5.3 Flow cytometry

PRP was activated with TRAP-6 (25 µM) and P-selectin and PAC-1 expression was assessed by flow cytometry, as described in section 2.2.9.

7.2.6 Rheometry

9ml aliquots of whole blood collected into Vacuette vacutainers without anti-coagulant were transferred directly and immediately (< 60 seconds) after sampling to a double-gap concentric cylinder geometry of a TA instruments Discovery HR-2 controlled stress

rheometer (New Castle, DE, USA). All work was conducted at 37(±0.1) °C and all measurements were made on aliquots of the same sample by use of similar measuring geometries with identical measuring surfaces and surface preparation procedures. The viscoelastic properties of incipient clots were determined by detecting the GP of samples of whole unadulterated blood using small amplitude oscillatory shear rheometry [366]. From GP the values of T_{GP} and d_f were measured and from d_f relative mass (RM) was calculated.

7.2.7 Scanning electron microscopy

For scanning electron microscopy (SEM) samples at each time point were allowed to clot at 37°C for a minimum of 15 mins. Samples were then washed three times with 2 cocadylate buffer pH 7.2 for the removal of excess salt and fixed for a minimum of 4 hrs in 2% glutaraldehyde solution. The clots were then rinsed with cocadylate buffer and dehydrated in a series of ethanol concentrations from 30 to 100%. The clots were then critical point dried with hexamethyldisilazane for 45 mins and placed in a fume hood for 24 hrs. Finally the clots were mounted to 0.5" SEM stubs (Agar Scientific, UK) and sputter coated with gold palladium. All samples were investigated with a Hitachi S4800 scanning electron microscope (Hitachi, High-Technologies Corporation, Tokyo, Japan) [372]

7.3 Results

7.3.1 Baseline fractal dimension (d_f) values and correlation with haemostatic and haematological coefficients

Analysis of pre-treatment blood indicated a clearly defined d_f value within a narrow range, representing a normal index of haemostasis where d_f = 1.72 (± 0.05) (Table 7.1). Minimum and maximum d_f were 1.63 and 1.91, respectively. Further analysis of pre- and post-treatment d_f values demonstrated a clear correlation with fibrinogen (0.037, p=0.04). There was no correlation between d_f and PT, APTT, haematocrit, platelet count or haemoglobin (Table 7.2).

Baseline d_f	**Value**
Minimum	1.63
25th percentile	1.69
Median	1.71
75th percentile	1.73
Maximum	1.91
Mean	1.72
Std. error of mean	0.05

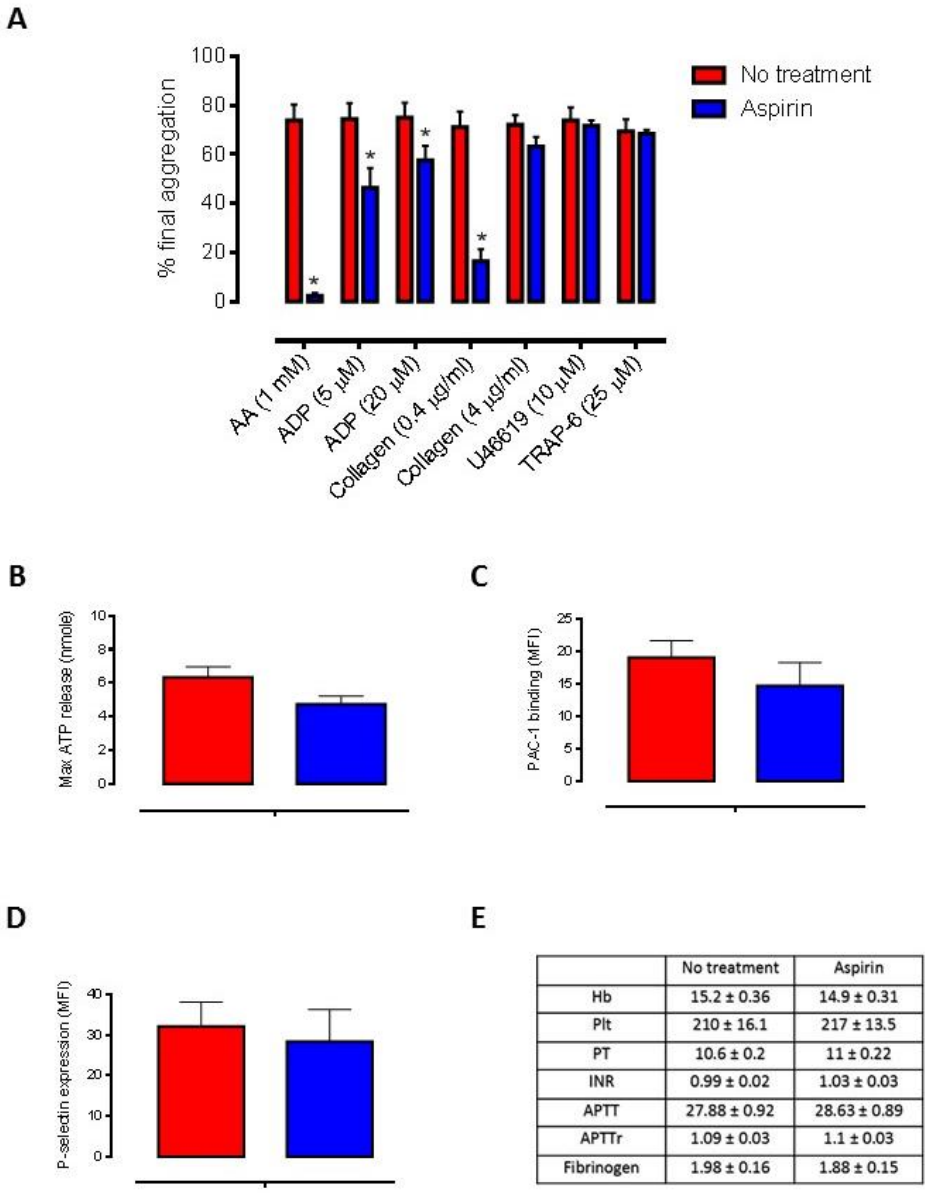
Table 7.1: Column statistics for baseline d_f values. d_f fractal dimension

Parameter	**Correlation coefficient**
Fibrinogen	0.376 *
PT	-0.301
APTT	0.154
Platelets	0.171
Haemoglobin	0.238
Haematocrit	0.252

Table 7.2: Pearson correlation coefficients for d_f versus haemostatic and haematological parameters. PT indicates prothrombin time; APPT activated partial thromboplastin time; d_f fractal dimension. * = Significant result (p<0.05)

7.3.2 Effects of aspirin (75 mg) therapy on platelet reactivity and fractal analysis of incipient clots

In individuals taking aspirin, standard LTA responses to AA (1 mM) and collagen (0.4 μg/ml) were strongly inhibited; 74%±6% to 2%±1% and 71%±6% to 16%±5%, $p<0.05$, respectively. Responses to ADP (5 μM) were also significantly reduced; 74%±6 to 46%±8, $p<0.05$. Those to ADP (20 μM), collagen (4 μg/ml), U46619 (10 μM) and TRAP-6 (25 μM) were unaffected (Figure 7.1.A). Aspirin therapy did not affect dense granule secretion induced by collagen (4 μg/ml) as measured by ATP release (6.3±0.63 to 4.73±0.5 nM, $p=0.11$) (Figure 7.1.B), or TRAP-6 (25 μM) induced PAC-1 binding (19±3 to 15±4 MFI, $p=0.40$) (Figure 7.1.C) or P-selectin expression (32±6 to 28±8 MFI, $p=0.73$) (Figure 7.1.D). Haematological parameters and selected haemostatic parameters were not altered (Table 7.1.E). Aspirin treatment also produced no significant changes in the value of d_f (1.71±0.01 to 1.69±0.01, $p=0.41$) or the RM (-8±19% change, $p=0.47$) of incipient clots (Figures 7.2.A and 7.2.B).



	No treatment	Aspirin
Hb	15.2 ± 0.36	14.9 ± 0.31
Plt	210 ± 16.1	217 ± 13.5
PT	10.6 ± 0.2	11 ± 0.22
INR	0.99 ± 0.02	1.03 ± 0.03
APTT	27.88 ± 0.92	28.63 ± 0.89
APTTr	1.09 ± 0.03	1.1 ± 0.03
Fibrinogen	1.98 ± 0.16	1.88 ± 0.15

Figure 7.1: Effects of aspirin (75 mg) therapy on platelet reactivity. Bar graphs representing platelet responses before and after treatment measured by: A) standard LTA to AA (1 mM), ADP (5 and 20 µM), collagen (0.4, and 4 µg/mL), TRAP-6 amide (25 µM) and U46619 (10 µM); B) ATP release after collagen (4 µg/ml) stimulation; C) TRAP-6 (25 µM) induced PAC-1 binding; D) TRAP-6 (25 µM) induced P-selectin expression and E) tabulated corresponding haematological and haemostatic parameters. Data are presented as final aggregation (%, mean±SEM), ATP release (nmol, mean±SEM) or geometric mean fluorescence index (MFI) (units, mean±SEM).

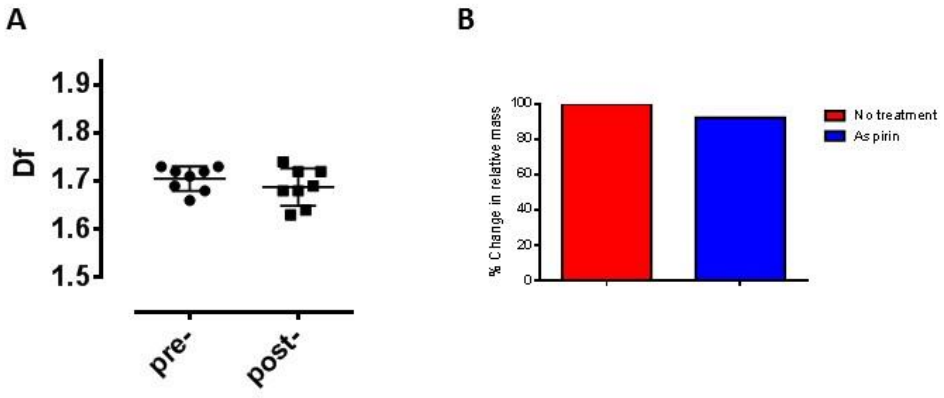
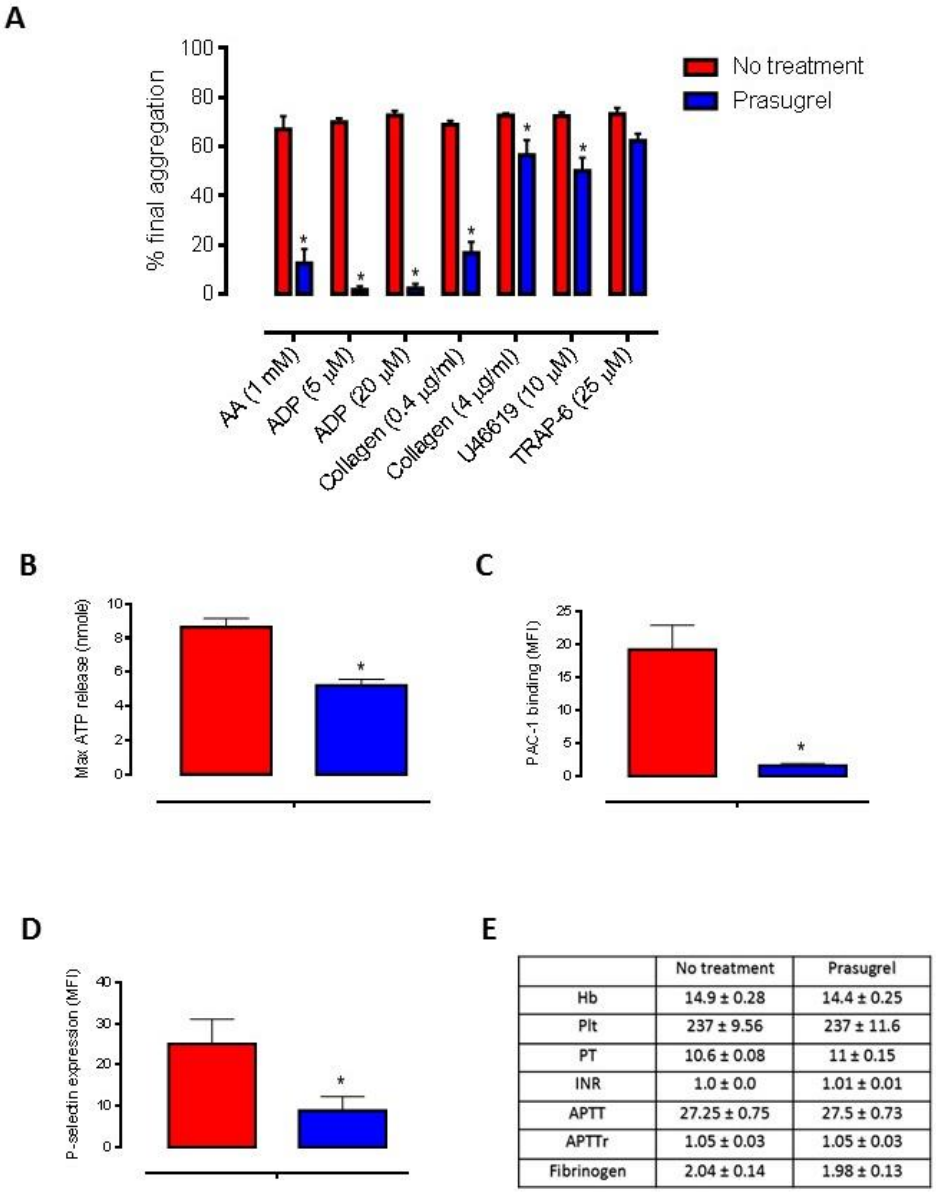


Figure 7.2: Effects of aspirin (75mg) on fibrin clot microstructure as calculated by viscoelastic properties of incipient clots. Changes in A) d_f and B) RM of incipient clots before and after treatment.

7.3.3 Effects of prasugrel (10 mg) monotherapy on platelet reactivity and fractal analysis of incipient clots

Following prasugrel therapy, aggregatory responses to AA (1 mM), collagen (0.4 and 4 µg/ml), ADP (5 and 20 µM), as well as U46619 (10 µM) were all significantly reduced. TRAP-6 (25 µM)-induced aggregation however, was unaffected (Figure 7.3.A). ATP release induced by collagen (4 µg/ml) was significantly decreased; 8.6±0.5 to 5.2±0.0.4 nM, $p<0.05$ (Figure 7.3.B). TRAP-6 (25 µM) induced PAC-1 and P-selectin expression were also both reduced; 19.2±3.7 to 1.6±0.3 MFI and 25±6.1 to 8.8±3.4 MFI, $p<0.05$, respectively (Figures 7.3.C and 7.3.D). Haematological and coagulation profiles remained unchanged (Table 7.3.E). There were notable reductions in d_f (1.72±0.02 to 1.67±0.01, $p =0.03$) and mean RM (-40±11% change, $p=0.03$) for incipient clots formed following prasugrel therapy (Figures 7.4.A and 7.4.B).



	No treatment	Prasugrel
Hb	14.9 ± 0.28	14.4 ± 0.25
Plt	237 ± 9.56	237 ± 11.6
PT	10.6 ± 0.08	11 ± 0.15
INR	1.0 ± 0.0	1.01 ± 0.01
APTT	27.25 ± 0.75	27.5 ± 0.73
APTTr	1.05 ± 0.03	1.05 ± 0.03
Fibrinogen	2.04 ± 0.14	1.98 ± 0.13

Figure 7.3: Effects of prasugrel (10 mg) therapy on platelet reactivity. Bar graphs of platelet responses measured by: A) standard LTA; B) ATP release after collagen (4 µg/ml) stimulation; C) TRAP-6 (25 µM) induced PAC-1 binding; D) TRAP-6 (25 µM) induced P-selectin expression and E) tabulated corresponding haematological and haemostatic parameters. Data are presented as final aggregation (%, mean±SEM), ATP release (nmol, mean±SEM) or geometric mean fluorescence index (MFI) (units, mean±SEM). Significance is shown as * $p<0.05$ vs non-treated throughout.

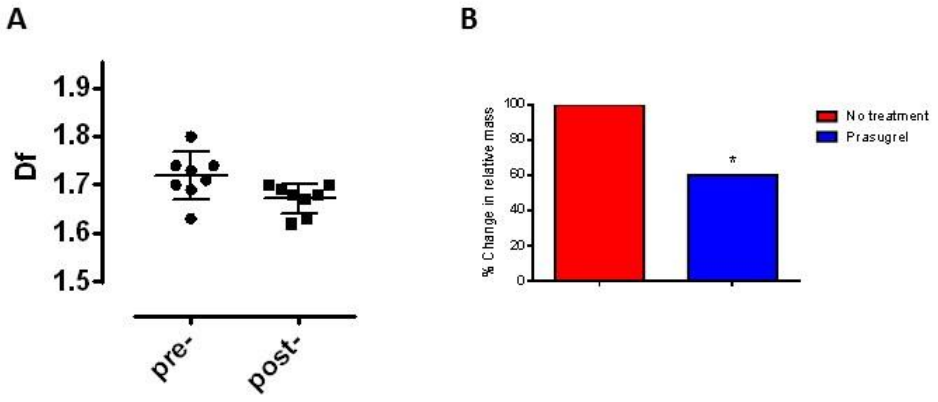
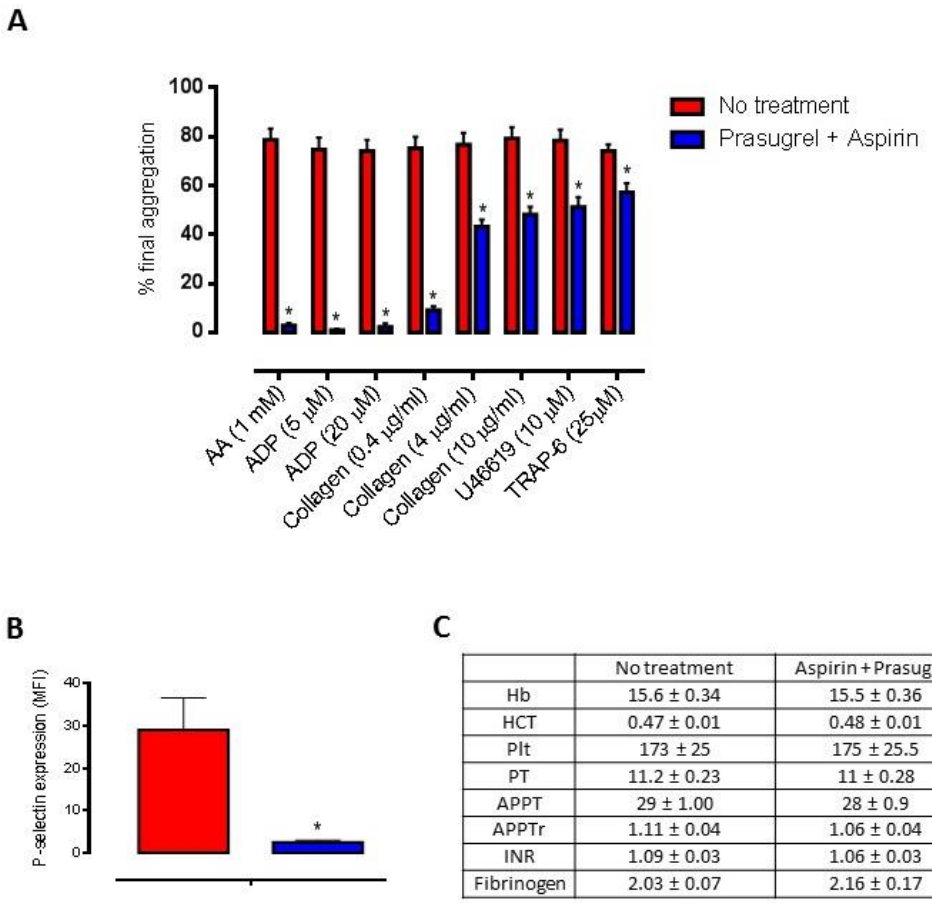


Figure 7.4: Effects of prasugrel (10mg) on fibrin clot microstructure as calculated by viscoelastic properties of incipient clots. Changes in A) d_f and B) RM of incipient clots before and after treatment.

7.3.4 Characterisation of DAPT with aspirin (75mg) plus prasugrel (10 mg) on platelet reactivity and fractal analysis of incipient clots

Platelet aggregation induced by AA (1mM), collagen (0.4 µg/ml) and ADP (5 µM and 20 µM) in LTA were abolished in individuals taking prasugrel plus aspirin. Responses to collagen (4 µg/ml), U46619 (10 µM) and TRAP-6 (25 µM) were also all significantly inhibited (Figure 7.5.A). TRAP-6 (25 µM) induced P-selectin expression was also decreased; 29±8% to 2±1%, $p< 0.05$ (Figure 7.5.B). No change was observed in haemostatic or haematological parameters (Table 7.5.C). There was a significant reduction in d_f value (1.73±0.02 to 1.68±0.02, p=0.03) for incipient clots and a resulting decrease in mean RM (-35±16% change, p=0.04) (Figure 7.6.A and 7.6.B).



	No treatment	Aspirin + Prasugrel
Hb	15.6 ± 0.34	15.5 ± 0.36
HCT	0.47 ± 0.01	0.48 ± 0.01
Plt	173 ± 25	175 ± 25.5
PT	11.2 ± 0.23	11 ± 0.28
APPT	29 ± 1.00	28 ± 0.9
APPTr	1.11 ± 0.04	1.06 ± 0.04
INR	1.09 ± 0.03	1.06 ± 0.03
Fibrinogen	2.03 ± 0.07	2.16 ± 0.17

Figure 7.5: Influence of DAPT with aspirin (75mg) plus prasugrel (10 mg) on platelet reactivity. Bar graphs demonstrating platelet responses before and after treatment measured by: A) standard LTA; B) TRAP-6 (25 µM) induced P-selectin expression and C) corresponding haematological and haemostatic parameters.

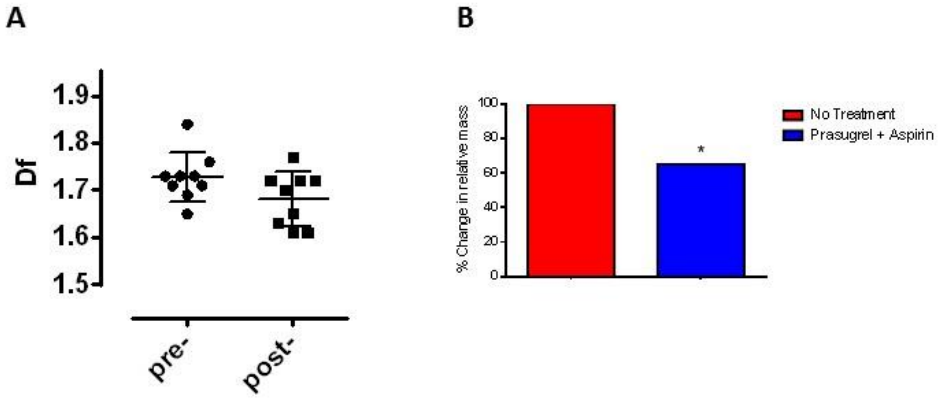
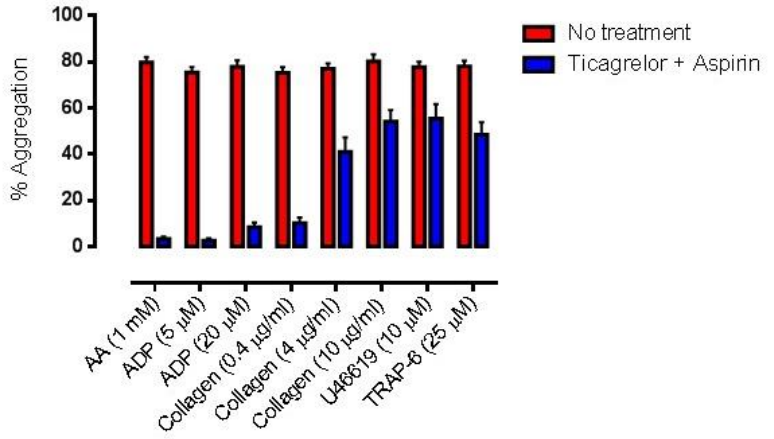


Figure 7.6: Effects of aspirin (75mg) plus prasugrel (10mg) on fibrin clot microstructure as calculated by viscoelastic properties of incipient clots. Changes in A) d_f and B) RM of incipient clots before and after treatment.

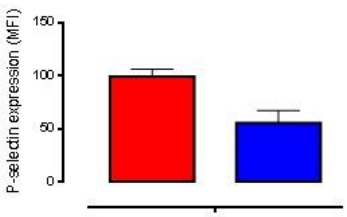
7.3.5 Characterisation of DAPT with aspirin (75mg) plus ticagrelor (90 mg) on platelet reactivity and fractal analysis of incipient clots

Following DAPT with aspirin plus ticagrelor platelet responses followed a similar pattern to those in individuals taking aspirin plus prasugrel; LTA aggregation induced by AA (1mM), collagen (0.4 µg/ml) and ADP (5 µM and 20 µM) were powerfully inhibited. Those to collagen (4 µg/ml), U46619 (10 µM) and Trap-6 (25 µM) were significantly reduced (Figure 7.7.A). These reductions were associated with a reduction in P-selectin expression ($p<0.05$) induced by TRAP-6 (25 µM); 99±7 to 55±12 (Figure 7.7.B). Haematological and coagulation profiles remained unchanged (Tables 7.7.C). There were significant decreases in d_f for incipient clots formed (1.72±0.03 to 1.63±0.02, p=0.04) and marked reductions in RM (-45±14% change, p=0.04) (Figure 7.8a and 7.8b).

A



B



C

	No treatment	Aspirin + Ticagrelor
Hb	14.8 ± 0.41	14.7 ± 0.37
HCT	0.42 ± 0.01	0.41 ± 0.01
Plt	208 ± 7.2	218 ± 7.5
PT	10.7 ± 0.14	10.7 ± 0.17
APPT	28.0 ± 0.42	26.8 ± 0.55
APPTr	1.09 ± 0.01	1.04 ± 0.02
INR	1.07 ± 0.02	1.03 ± 0.02
Fibrinogen	2.05 ± 0.23	2.10 ± 0.14

Figure 7.7: Influence of DAPT with aspirin (75mg) plus ticagrelor (90 mg) on platelet reactivity. Bar graphs depicting platelet responses prior to and following treatment as measured by: A) standard LTA; B) TRAP-6 (25 µM) induced P-selectin expression and corresponding haematological and haemostatic parameters.

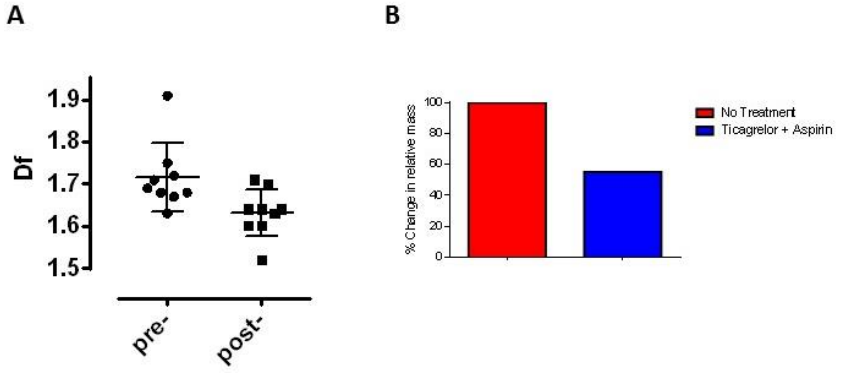


Figure 7.8: Effects of aspirin (75mg) plus ticagrelor (90mg) on fibrin clot microstructure as calculated by viscoelastic properties of incipient clots. Changes in A) d_f and B) RM of incipient clots before and after treatment.

7.3.6 Clot structure analysis by SEM and fractal analysis

SEM demonstrated that DAPT resulted in formation of clots with reduced fibrin density and increased fibrin strand diameter (Figure 7.9). This reduction in clot density was consistent with the d_f measurements and projections of RFAs for the aspirin plus prasugrel and aspirin plus ticagrelor groups showing a reduction in the density of connectedness compared to that projected from blood collected under control conditions (Figure 7.10).

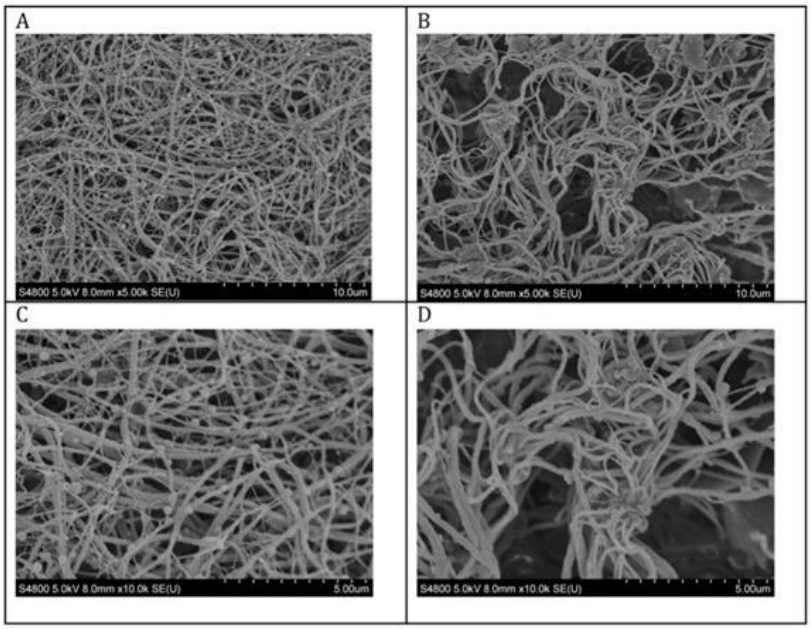


Figure 7.9: Influence of DAPT with aspirin (75 mg) plus ticagrelor (90 mg) on clot structure. Corresponding SEM micrographs of clots formed in blood taken from one participant, before (panels A and C) and after (panels B and D) treatment with aspirin (75mg) plus ticagrelor (90

mg). Micrographs were captured at two different magnifications, 5K (panels A and B) and 10K (panels C and D), to visualize the organisation of clot structure. Sizing bars are along the bottom edges of the images.

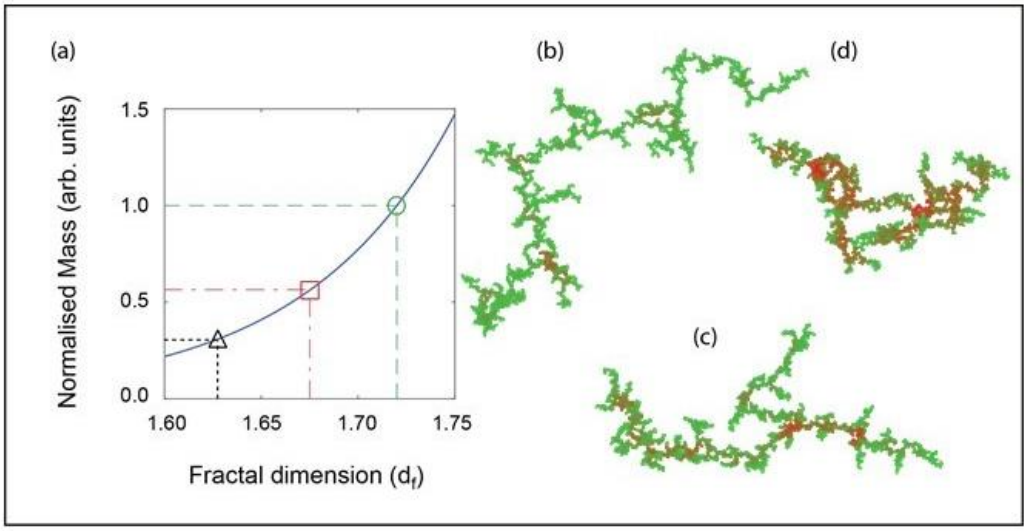


Figure 7.10: Visualisation of the relationship between d_f and mass. Figure (A) indicates the variation of the mass of a random fractal aggregate (RFA) as a function of d_f. The magnitude of the RFA is normalized to the mean healthy value, d_f = 1.72 (green circle). Mass values of RFA where d_f = 1.68 (red square) and d_f = 1.63 (black triangle) represent, respectively, the effects of aspirin + prasugrel and aspirin + ticagrelor. Figures (b), (c) and (d) are numerical realisations of RFAs with fractal dimensions of 1.63, 1.68 and 1.72 (normal), respectively. Each numerical realisation of RFA is comprised of a number of constituent particles of unit diameter all of which are connected to at least one other particle. The colour index in the RFA realisations represents the local densities of constituent connected particles (green and red shades represent low and high density regions, respectively). A RFA realisation containing many red areas denotes a more compact, densely connected structure than a RFA with predominately green areas.

7.4 Discussion

Here, advanced rheology has demonstrated the key importance of platelets as regulators of clot microstructure under clinically relevant conditions. The measurement of d_f was used to quantify differences in microstructural organisation and mechanical properties of blood clots secondary to individual and combined anti-platelet therapy regimes.

Analysis of pre-treatment blood indicated a clearly defined d_f value within a narrow range, representing a normal index of haemostasis where d_f = 1.72 (± 0.05). Further analysis of pre and post treatment d_f values demonstrated a clear correlation with fibrinogen (0.037, p=0.04), supporting the association of the microstructural characteristics of the fibrin clot with d_f. For platelet studies high pre-treatment levels of platelet reactivity were observed, as is normal in healthy volunteers [244, 323, 324].

Following treatment with aspirin or prasugrel there was powerful inhibition of platelet responses to AA or ADP, respectively, in keeping with consensus statement indicators of effective therapy [244, 324]. Aspirin monotherapy also inhibited platelet aggregations induced by low concentrations of ADP and collagen. ATP release, activation of GP IIb/IIIa receptors and P-selectin expression however, were not significantly affected. In accordance with these limited effects on platelet function, aspirin produced no significant change in clot microstructure as detected by d_f (1.71±0.01 to 1.69±0.01, p=0.41). The change in mean RM was also non-significant (-8±19% change, p=0.47). Prasugrel monotherapy substantially blunted platelet aggregation responses to all agonists tested apart from TRAP-6 and caused significant reductions in ATP release, P-

selectin expression and GP IIb/IIIa receptor activation. Platelet inhibition was associated with both a significant reduction in d_f (1.72±0.02 to 1.67±0.01, p =0.03) and a considerable change in the mean RM of incipient clots (-40±11% change, p=0.03) indicative of a substantial change in microstructure.

Following the monotherapy studies, which indicated a strong association between platelet activity and clot architecture, the next step was to characterise the influences of DAPT. Administration of aspirin plus prasugrel or aspirin plus ticagrelor resulted in significant levels of platelet inhibition to all agonists, abolishing responses to weaker agonists tested. P-selectin expression was also significantly reduced in both groups. Again, significant platelet inhibition was coupled with a reduction in d_f: aspirin plus prasugrel (1.73±0.02 to 1.68±0.02, p=0.03) and aspirin plus ticagrelor (1.72±0.03 to 1.63±0.02, p=0.04). There were also significant reductions in mean RM both for aspirin plus prasugrel (-35±16% change, p=0.04) and for aspirin plus ticagrelor (-45±14% change, p=0.04).

Overall, these results clearly demonstrate that stronger inhibition of platelet function is associated with the formation of less dense clots comprised of a more open/porous network of fibrin strands. It is important to recognise that d_f has a very narrow range and to appreciate the non-linear relationship between d_f and fibrin mass. Figure 7.10 helps in this regard by providing a visual projection of how relatively small changes in d_f would underlie large changes in RFA; notably the lowest d_f has a lower frequency of high-density regions (i.e. the number of nearest neighbours is relatively low) consequently the projected form is relatively sparse and open which would equate to a mechanically weaker and more highly friable clot. On the contrary, the RFA with the

highest d_f is relatively more compact, due to the larger number of high density regions within the structure.

This predicted link between d_f and thrombus structure is confirmed by SEM, which demonstrates a more open clot structure following treatment with anti-platelet therapies. These SEM images highlight that incipient clot structure quantified by d_f acts as a template to predict the structure of the mature clot. Notably, the number of small round particles, which represent platelets, is much reduced between the pre- and post-treatment images. This is in keeping with reduced platelet-fibrin interactions which, I would expect following DAPT with aspirin plus ticagrelor in the healthy volunteer subject.

These observations support the idea that indices of fractal analysis are intricately linked and reflect clot microstructure and strength and whilst these are determined by multiple components, are representative of the dynamic and interwoven properties of the fibrin clot and platelet reactivity. Importantly, the experiments reported here show that this technique is able to quantify the effects of individual therapeutic interventions. Comparison of responses in the absence and presence of different levels of platelet inhibition identifies a significant relationship between platelet function and the complex, highly disordered microstructure of incipient clots in whole unadulterated blood from healthy volunteers. These results are readily understandable as as highlighted in chapter 1, platelets are central to several key coagulation processes.

The results in this chapter, in keeping with previous studies suggest that this novel point of care visco-elastic technique is able to accurately and reproducibly assess clot

architecture with the potential of determining the effects of disease and therapeutic intervention on clot quality. This technique could be applied to investigate the functional relationship between coagulation pathways and platelets as a potentially more globally representative marker of haemostasis and thrombosis.

Chapter 8: General discussion

8.1 Discussion

This thesis explores the hypothesis that the level of platelet reactivity during DAPT will be a function of the level of $P2Y_{12}$ receptor blockade and the levels of endothelial produced NO and PGI_2 in individual patients because of the strong synergistic interactions between these three mediators. I show firstly *in vitro* the powerful potentiation by $P2Y_{12}$ receptor blockade of the recognised synergy between NO and PGI_2. I then show in healthy individuals and patients with cardiovascular disease receiving a $P2Y_{12}$ receptor blocker as monotherapy or as DAPT, that *ex vivo* responses to the strong primary platelet activators collagen and TRAP-6 are powerfully influenced by the presence of NO and PGI_2. The strong synergies between $P2Y_{12}$ inhibitors and the cAMP and cGMP signaling systems mean that *in vivo* platelet reactivity in patients receiving $P2Y_{12}$ receptor blockers as part of DAPT will vary in accordance with endogenous levels of endothelial-derived NO and PGI_2. This provides an explanation for different thrombotic outcomes in the presence of similar levels of platelet blockade; i.e. individual patients with different levels of endothelial function, or indeed disease-driven endothelial dysfunction, would have different levels of *in vivo* platelet inhibition for the same level of DAPT activity, as determined by *ex vivo* testing.

DAPT, aspirin plus a $P2Y_{12}$ receptor blocker, is the preventative therapy provided to patients at particular risk of coronary thrombosis, notably for the first 12 months following coronary stent implantation or an ACS [127, 260]. Despite this therapeutic approach coronary thrombosis still occurs, and there have been great efforts made to find *ex vivo* tests that could predict for clinical outcomes [266, 267]. Deductive reasoning leads to the conclusion that less effective platelet blockade would leave individuals at

increased risk of thrombosis and so multiple efforts have been made to link levels of platelet reactivity in *ex vivo* tests to clinical outcomes. Despite the attractive logic of this approach, tailoring anti-platelet therapy to *ex vivo* platelet responses has failed to provide any improvement in clinical outcomes, as noted in large scale studies such as ADRIE [274] and several large-scale, prospective RCT, such as GRAVITAS [275], ARCTIC [276], TRIGGER-PCI [277] and TRILOGY [278] .

In patients receiving clopidogrel, there are well-characterised metabolic differences that can produce suboptimal levels of its active metabolite and consequently result in suboptimal levels of $P2Y_{12}$ receptor blockade [262]. There are also some reports of variability in the effects of prasgurel and ticagrelor, although to a much lesser extent than for clopidogrel [144]. Biochemical resistance to the effects of aspirin are also particularly rare [223]. Allowing for differences dependent upon adherence to therapy, individuals on DAPT may in fact present rather a more homogenous level of platelet inhibition than can be associated to different clinical outcomes. Appreciating that blockade of $P2Y_{12}$ receptors greatly increases the inhibitory effects of NO [238], and knowing that not only is there a similar interaction with the inhibitory effects of PGI_2 [237] but that NO and PGI_2 powerfully synergise to inhibit platelets [74], one can reason that differences in the levels of NO and PGI_2 in the presence of the same levels of $P2Y_{12}$ receptor blockade would produce different levels of platelet inhibition. By testing this hypothesis firstly *in vitro* and then in individuals receiving $P2Y_{12}$ blockers, results reported here show that strong and synergistic interactions between $P2Y_{12}$ receptor blockade and endothelial derived mediators produce profound inhibitory effects upon platelets.

The *in vitro* studies presented firstly highlight the strong amplification by $P2Y_{12}$ blockade on the inhibitory effects of both PGI_2 and NO individually and then secondly demonstrate the even more dramatic inhibitory effects of all three interacting to produce profound platelet inhibition. Blockade of platelet $P2Y_{12}$ receptors dramatically enhances the anti-platelet potency of NO and PGI_2 over a range of concentrations, causing over a 1,000-fold increase in their inhibitory activity against platelet aggregation. Notably, these powerful interactions between NO, PGI_2 and $P2Y_{12}$ receptor blockade in inhibiting platelets are markedly synergistic, as noted by isobolographic analysis. Utilizing very low concentrations of NO and PGI_2 in the nanomolar range I further demonstrated this powerful potentiation through platelet aggregation and ATP release experiments in response to activation of receptors for ADP, thrombin and collagen. To then make data readily accessible results have been presented in the form of heat maps that move from red to green, indicating movement from full platelet activation to no platelet activation. These results demonstrate that even in the context of 100% $P2Y_{12}$ blockade when neither NO or PGI_2 are present both collagen and TRAP-6 stimulation still result in high levels of platelet aggregation. Aggregation is reduced in the presence of both inhibitors individually but virtually abolished with NO and PGI_2 acting in combination with $P2Y_{12}$ blockade. In accordance with this, marked reductions in platelet activation were recorded in ATP release experiments with the potentiation of the actions of both NO and PGI_2 by $P2Y_{12}$ blockade. Interestingly, the addition of aspirin to PAM in the absence of endothelial mediators reduced aggregation induced by collagen but not by TRAP-6. Aspirin however, added little additional inhibitory effect to platelet inhibition resulting from the synergistic interactions of $P2Y_{12}$ blockade, NO and PGI_2.

An *in vitro* approach was also used to model events in the presence of suboptimal levels of $P2Y_{12}$ receptor blockade by using concentrations of PAM that were 50% and 25% of the effective concentration. Under these conditions it was noted that relative to consensus levels of DAPT there were no significant reductions in platelet aggregation. Notably, however, in the presence of NO and PGI_2, effective levels of inhibition were achieved, even when platelets were exposed to only 25% of the effective concentration of PAM. As expressed in heat maps, there is a clear interaction between PAM, DAPT and the endothelial mediators that move platelets from reactive ('red') to unreactive ('green'). Interestingly, these comparisons indicate that 25% of the effective concentration of PAM plus NO and PGI_2 produces a stronger inhibition in LTA, the 'gold standard test', than 100% of the effective concentration of PAM in the absence of NO and PGI_2 (i.e. the normal conditions for testing *ex vivo* platelet responsiveness). This suggests that in individuals in whom suboptimal $P2Y_{12}$ inhibition is achieved, such as poor clopidogrel metabolisers, anti-platelet efficacy may be particularly sensitive to any changes in endothelial function. The *in vitro* data also demonstrates that the triple synergy between $P2Y_{12}$ blockade, NO and PGI_2 can be explained by changes in cAMP signaling which, is consistent with the known interactions between NO and PGI_2 [322] and PGI_2 and $P2Y_{12}$ [237]. cGMP was not increased even in the optimal condition of $P2Y_{12}$ receptor blockade, NO and PGI_2. For both primary platelet agonists tested, cAMP was not affected by the combination of NO and PGI_2 or $P2Y_{12}$ individually but significant elevations were noted when all three were allowed to interact. These interactions were further supported by measurements of phospho (Ser^{239})-VASP, a downstream marker of PKG and PKA activation and therefore, representative of cyclic nucleotide activity within platelets. PAM treatment resulted in elevations of VASP-phosphorylation in all treatment conditions tested following TRAP-6 stimulation but notably the highest

increase was observed, again, in the presence of $P2Y_{12}$ blockade, NO and PGI_2. Of note, aspirin treatment alone did not produce any increase in VASP phosphorylation in any of the conditions tested.

To further test the overarching hypothesis, I then sought to ascertain whether these *in vitro* interactions held true in man. I did this by analysing platelet behaviour *ex vivo* using several tests of platelet activity in healthy volunteers prescribed monotherapy aspirin or prasugrel or the two in combination, prescribed as DAPT. Firstly, it was established that the drug regimes used in the studies elicited satisfactory reductions in baseline reactivity in the LTA therefore, establishing effectiveness of $P2Y_{12}$ and/or COX inhibition in accordance with suggested analytical cutoffs [324]. These reductions were against high pre-treatment levels of platelet reactivity (>70% response to 5 μM ADP) [123]. In these studies and others presented here, care was taken to include the standard measures of platelet function as determined in consensus statements [324, 325]. In volunteers taking prasugrel or aspirin there were inhibitions of responses to ADP or AA, respectively that were in keeping with consensus statements of effective therapy. For DAPT volunteers both AA and ADP were also appropriately inhibited; i.e. the drugs were working to an effective level of clinical efficacy. Despite this level of effective inhibition, high concentrations of the strong primary platelet activators, TRAP-6 or collagen, still caused notable platelet activation in all three treatment groups. Addition of low concentrations of NO and PGI_2 had moderate effects on their own following TRAP-6 stimulation in both prasugrel and DAPT treated volunteers but no effect in those taking aspirin. In combination to model the environment within the blood vessel, PGI_2 and NO led to almost complete inhibition in platelets from individuals treated with DAPT or prasugrel. In the aspirin group levels of platelet inhibition were much more modest. TRAP-6 is a

strong platelet agonist and even at high concentrations the powerful synergy between $P2Y_{12}$ blockade, NO and PGI_2 remarkably, completely eliminated platelet activation. An effect which was notably not associated with aspirin therapy. Whilst PGI_2 plus NO was associated with reductions in all three treatment groups following collagen stimulation, this effect was most marked in the DAPT group and least so in aspirin treated volunteers. The modest inhibitory effect associated with aspirin therapy can be explained by the synergy between PGI_2 and NO acting alone in the context of relatively weak platelet stimulation with low dose collagen. Similarly, in ATP release experiments, in the context of DAPT, NO or PGI_2 alone had modest effects upon platelet granule release however, when combined they caused more than 50% inhibition.

These results indicate that even in the presence of effective DAPT, i.e. within consensus guidelines, the presence of NO and PGI_2 lead to very much higher levels of platelet inhibition. The powerful interaction between $P2Y_{12}$ blockade, NO and PGI_2 was further supported by quantification of P-selectin and PAC-1 expression, two markers of platelet activation. Whilst expressions of both of these markers were reduced by $P2Y_{12}$ blockade alone in both prasugel and DAPT groups, the addition of NO or PGI_2 caused further significant platelet inhibition and the combination of PGI_2 and NO abolished P-selectin and PAC-1 activation. Notably, neither P-selectin nor PAC-1 expression were reduced by aspirin therapy with or without the addition of NO or PGI_2 individually or in combination. Having shown through several tests of platelet activation that the powerful antithrombotic effects of $P2Y_{12}$ receptor blockers are mediated by profound potentiation of the endogenous synergy between NO and PGI_2 *ex vivo,* in a similar manner to that demonstrated in *in vitro* experiments, I sought to establish whether this powerful synergism was also explained *ex vivo* by blockade of $P2Y_{12}$ receptor-dependent

cyclic nucleotide pathways of platelet activation. Again, I was able to demonstrate that cAMP is an important signaling molecule and downstream effector of the synergistic actions of $P2Y_{12}$ blockade, NO and PGI_2. This interplay however, was not related to altered synthesis or degradation of cGMP. I observed this with two separate $P2Y_{12}$ receptor blockers, prasugrel and ticagrelor after activation with thrombin or collagen. Thus far, these experiments suggest that the true inhibitory potential of the synergy of NO and PGI_2 is only revealed by blockade of $P2Y_{12}$ receptors and that activation of this receptor has the ability to limit the powerful anti-platelet effects of NO and PGI_2.

Having demonstrated *in vitro* and *ex vivo* in healthy volunteers that after strong activation $P2Y_{12}$ receptor blocked platelets, as determined by aggregation and ATP release experiments, as well as by VASP phosphorylation, P-selectin, PAC-1 and cyclic nucleotide activity, are able to sense nanomolar concentrations of PGI_2 and NO acting in combination, I sought to establish whether these interactions take place to the same degree in disease states. In order to do this I studied platelets from patients with PAD, a disease associated with high morbidity and mortality [328] and interestingly, a patient group with an unexplained poorer prognosis following ACS [331]. For these reasons, I studied platelets from both treatment naïve patients and from patients prescribed a range of anti-platelet therapies, namely aspirin, clopidogel or aspirin plus clopidogrel as DAPT.

I did not find any evidence that platelets from treatment naïve patients with PAD were more reactive *in vitro* compared to those from healthy volunteers, as assessed by two methods of platelet aggregation using several platelet agonists. This is suggestive that it is the *in vivo* environment that is key in determining platelet reactivity and therefore,

reduction in production of inhibitory endothelial mediators, associated with the diffuse endothelial dysfunction that occurs in this disease, could lead to increased platelet reactivity *in vivo* in these patients. This reduced bioavailability of NO and PGI_2 could also reduce the efficacy of $P2Y_{12}$ receptor antagonists by limiting the potency of their synergistic relationship with $P2Y_{12}$ blockade. This could partly explain the poorer outcomes in this population following ACS despite appropriate DAPT therapy.

There were higher levels of AA induced aggregation in platelets from patients taking aspirin compared to healthy volunteers prescribed aspirin for a week (15±6% versus 2±1%, p=0.156). Although this difference was not found to be significant it could be indicative of non-adherence to therapy, elevated platelet turnover or reduced aspirin efficacy, which could increase thrombotic potential in this patient group. Aggregation was not significantly changed in response to any of the other agonists tested. I also tested platelet responses to NO plus PGI_2 to both primary platelet agonists, TRAP-6 and collagen. I found that the combination of PGI_2 and NO was associated with the lowest levels of platelet aggregation following collagen stimulation in all therapy groups tested. Of note, the effects of endothelial mediators were significantly more profound in clopidogrel and DAPT treated patients, indicative that the underlying three-way synergy between NO, PGI_2 and $P2Y_{12}$ blockade is present and effective in patients with PAD prescribed $P2Y_{12}$ antagonist therapies at standard doses. Following stimulation with the stronger platelet agonist TRAP-6, PGI_2+NO only significantly reduced aggregation in clopidogrel monotherapy and DAPT treated patients. Again with this second agonist tested, further evidence was found of the aforementioned synergy with significantly more profound platelet inhibition produced in the presence of $P2Y_{12}$ blockade with clopidogrel or DAPT, in contrast to aspirin which did not reduce aggregation as

compared to treatment naïve patients (naïve, 59±8; aspirin, 59±4%; clopidogrel, 36±9% ($p<0.05$); DAPT, 31±12% ($p<0.05$)). These experiments confirm that in PAD, a disease associated with atherosclerosis and atherothrombosis, high levels of platelet aggregation are still observed *ex vivo* following stimulation with the primary agonists TRAP-6 and collagen despite anti-platelet therapies and that *in vivo* if present, PGI_2 and NO could greatly influence platelet activity leading to far greater levels of platelet inhibition.

These *in vitro* and *ex vivo* studies in both healthy volunteers and patients with cardiovascular disease demonstrate the well-known synergy of NO and PGI_2 in inhibiting platelets [74] and the ability of $P2Y_{12}$ antagonism to potentiate the inhibitory actions of both PGI_2, dependent upon cAMP [237], and NO, dependent upon cGMP generation [238]. Furthermore, the three way synergy between these three inhibitors is many more times more powerful than the synergy existing between any combination of two inhibitors acting in pairs and provides evidence to support the hypothesis that *in vivo* the efficacy of $P2Y_{12}$ blockade will be highly influenced by levels of NO and PGI_2 and that *in vivo* platelet function will be a product of both internal platelet reactivity and the environmental influence of the endothelium. Though not widely commented upon, the body contains many more endothelial cells than platelets, in the order of 50 times more, and the two populations constantly interact meaning that within the circulation DAPT will exert its effects upon platelets in the presence of endothelial-derived mediators. Before going on to describe the therapeutic and diagnostic implications of these key interactions I will highlight the potential importance in the process of atherothrombosis. As shown here and in previous studies the inhibitory effects of NO and PGI_2 are severely blunted by activation of platelet $P2Y_{12}$ receptors by ADP. The observation that $P2Y_{12}$

activation can attenuate this powerful synergy suggests that following strong platelet stimulation, activation of $P2Y_{12}$ receptors is a principal mechanism that enables platelets to overcome the effects of endothelial inhibitory mediators [74] allowing for platelet activation and thrombus formation. Considering the strong inhibitory tone conferred upon platelets by endothelial cells, the endogenous intraplatelet pathways and key importance of $P2Y_{12}$ receptors described above, it is interesting to give some consideration into how it is that fast flowing platelets are able to aggregate at sites of arterial injury. It may well be that platelets carry their cyclic nucleotide tone with them from their passage through capillary beds and are also exposed to circulating levels of both PGI_2 and adenosine that will continue to elevate levels of cAMP. Platelets appear to be able to overcome this strong inhibitory tone through release of secondary mediators by the first arriving platelets most notably, ADP that activates $P2Y_{12}$ receptors on platelets. Crucially then, $P2Y_{12}$ receptor activation rapidly leads to a blockade of AC which turns off cAMP production and counteracts the inhibitory actions of cGMP. These concepts put the relationship between the endothelium and platelets at the centre of atherothrombosis. Endothelial cells produce NO and PGI_2 that together with adenosine and others elevate cAMP and cGMP in platelets and these two systems synergise to produce the greatest inhibition of platelet reactivity. This effect likely largely takes place within capillary beds that make up the majority of the cardiovascular system, not the large vessels, but rapidly moving platelets carry the effects of endothelial cell exposure with them. Exposure of platelets to a damaged blood vessel wall releases ADP that through $P2Y_{12}$ receptor activation pivotally turns off the cAMP and cGMP generating and signalling systems and in concert with very active phosphodiesterases rapidly switches the platelet to being strongly reactive.

The concept that the efficacy of $P2Y_{12}$ receptor antagonists will vary markedly as a function of endothelial activity in individual patients begs the question as to how anti-thrombotic therapies can best be optimised in particular individuals or patient groups with cardiovascular disease. Therapeutically, should we consider optimising the availability and activity of endothelial-derived mediators (such as by the co-administration of PDE inhibitors), or providing mimetic drugs, rather than adding in further anti-platelet therapies? The data reported here suggest that cAMP rather than cGMP is the major driver of this synergy so phosphodiesterase 3 (PDE3) inhibitors, such as cilostazol, may have an enhanced effect compared to PDE5 inhibitors, such as sildenafil or dipyridamole. Interestingly, several clinical studies have suggested that the addition of cilostazol to DAPT may be beneficial. In a small study of patients with complex lesions undergoing PCI cilostazol reduced MI (1.6% vs 13.6%, p=0.018) and MACE (1.6% vs 16.7%, p=0.004) respectively, without increasing bleeding [373]. Treatment with cilostazol was also found to be superior to DAPT in STEMI patients, notably procuring additional benefits in diabetic and older patients [374]. Both the DECARE-DIABETES and LONG trials also favourably associated add on cilostazol therapy to DAPT which was shown to improve patient outcome [167, 375]. These findings were reinforced by the results of a meta-analysis of RCT indicating that the addition of cilostazol significantly reduced the incidence of MACE and both target vessel and lesion revascularization in patients with IHD [376]. Authors of these studies state that the mechanisms behind the benefit of additional cilostazol are not fully understood. Proposed reasons are the pleiotrophic effects of cilostazol preventing the progression of atherosclerosis and a possible beneficial impact of cilostazol on plasma lipid profiles. It may be however, that the interaction between $P2Y_{12}$ receptor blockade and cilostazol elevates cyclic nucleotide levels in platelets leading to much enhanced levels of platelet

inhibition to reduce thrombotic risk and improve outcome. Interestingly, a similar role for PDE5 inhibitors which increase cGMP in platelets was not suggested in the ACCEL-DIP study with the addition of dypridamole to DAPT not reducing the prevalence of HPR [377]. This is in keeping with our recent finding that the synergy between $P2Y_{12}$ blockade, NO and PGI_2 is mostly cAMP dependent [239]. These findings could, eventually, be applied clinically in a personalised medicine framework where endothelial mediator production of individuals is assessed and appropriate add-on therapy applied. In a more generalised approach these additional therapies could also be supplied to patient groups with known endothelial dysfunction, such as diabetics. This approach could provide increased anti-platelet efficacy while avoiding the increased risk of bleeding events associated with the approach of triple anti-platelet therapy (TAPT).

The emergence of this complex and very powerful synergy also has additional therapeutic implications for DAPT. Therapeutically, it provides us with even further cause to question the benefit of addition of aspirin to strong $P2Y_{12}$ receptor blockade [148, 378]. As was outlined in the introduction of this thesis, in addition to its effects upon platelets, aspirin will also inhibit COX at other sites in the body and even at low, anti-thrombotic doses, aspirin produces substantial inhibition of COX within the vasculature, both in platelets and in the blood vessel wall. Crucially, reduction in vascular PGI_2 production through COX inhibition in endothelial cells can reduce platelet cAMP, increase platelet reactivity and so increase the potential for thrombosis. The incremental increase in platelet inhibition provided by aspirin inhibiting TXA_2 production on top of strong $P2Y_{12}$ receptor blockade may well be insufficient to balance this thrombogenic effect, so the addition of aspirin could effectively have a net negative

influence on the cardiovascular system. Furthermore, inhibition of the production of PGI_2 will lessen its participation and attenuate the powerful three-way synergy that we describe to increase platelet reactivity beyond that predicted for loss of PGI_2 alone. So while consideration of platelet activation pathways in isolation could lead to the conclusion that the addition of aspirin to strong $P2Y_{12}$ receptor blockade increases platelet inhibition, consideration of platelet activation pathways acting together *in vivo,* within the milieu of the circulation with the powerful influence of the endothelium may lead to the conclusion that the addition of aspirin to strong $P2Y_{12}$ blockade could actually decrease platelet inhibition.

Interestingly, as mentioned previously the role of aspirin in DAPT is being re-evaluated in clinical trials and has already been questioned in the context of TAPT with warfarin following publication of the WOEST study [379]. If aspirin were proven to provide little additional benefit to strong $P2Y_{12}$ receptor blockade, then drugs such as prasugrel or ticagrelor alone could become standard of care. DAPT could then become a strong $P2Y_{12}$ receptor blocker plus another drug; as suggested above, enhancing inhibitory cyclic nucleotide pathways in platelets could represent an efficacious line of therapy. In addition to the use of PDE inhibitors this could potentially be achieved through use of other drugs that increase platelet cyclic nucleotide levels such as synthetic PGI_2 analogues or direct GC activators. The use of some of these agents has previously been restricted due to negative effects upon the blood vessel wall for example, causing hypotension. However, due to the enhancing effects of $P2Y_{12}$ receptor blockade upon the inhibitory potencies of these drugs it is possible that these agents could be provided at doses that have more pronounced effects upon platelets with lesser of the previously reported limiting effects on the vasculature. By focusing on promoting these

endogenous inhibitory mechanisms rather than attempting to further inhibit pro-aggregatory pathways in platelets through the addition of a third anti-platelet agent, this newer DAPT approach could diminish platelet excitability and so reduce thrombosis without increasing the risk of bleeding.

As a diagnostic implication of these synergistic interactions, an estimation of endothelial function alongside *ex vivo* platelet testing could enhance thrombotic risk prediction in individual patients. As discussed, NO and PGI_2 markedly affect the potency of platelet inhibition by $P2Y_{12}$ receptor antagonists while these are absent in *ex vivo* testing, providing an explanation for the disconnect which exists between platelet testing and thrombotic outcomes in patients. Adjuvant endothelial assessment could improve the usefulness of PFT in influencing therapeutic management, helping to identify those individuals who truly are at risk of thrombosis and may benefit from additional drug intervention in a personalized therapeutic manner.

As a step towards the goal of measuring endothelial function in those patients with cardiovascular disease undergoing PFT and determining platelet activity as a product of these two factors, I pilot tested two methods of assessing endothelial function in healthy volunteers. Laser Doppler associated with iontophoresis and RH were selected as these methods are thought to assess the microcirculation and responses recorded in the skin microvasculature have been shown to be reflective of the responses taking place in other vascular beds [316]. Furthermore, these responses are thought to be under the influence of both PGI_2 and NO production. RH following a period of occlusion will reflect NO and prostanoid bioavailability and administration of ACh and SNP are representative of endothelial-dependent vasodilatation dependent on NO and prostaglandin

participation and smooth muscle dependent on non-endothelial pathways of vasodilation, respectively. Both techniques were well tolerated and despite intial difficulties in optimising the protocols due to lack of standardisation of both techniques, both gave accurate and reasonably reproducbile results, in keeping with published studies in healthy volunteers. Notably, I observed equal responses to ACh and SNP as would be expected in healthy volunteers lacking endothelial dysfunction and appropriate maximim response to PORH.

Of the two, I found PORH to be a more reliable technique as it gave the more reproducible results, was more time efficient and an easier technique to carry out. Iontophoresis with laser Doppler has in published studies been associated with poor reproducibility due to differences in skin resistance, spatial heterogeneity of skin blood flow and movement artefacts. However, as a technique it is possibly more sensitive and has the added benefit of being able to detect mild deficiencies in vascular response through administration of agonists at low and consequently graded current increments. This was certainly the more difficult technique to optimise however, substantial advances were made during the pilot period through minimisation of nonspecific, galvanic responses by current optimisation, choice of vehicle and reduction of spatial heterogeneity through careful and precise chamber placement. Therefore, this technique does hold promise. Certainly both techniques are attractive for the further exploration of the hypothesis that the efficacy of $P2Y_{12}$ receptor antagonists will vary in individual patients in accordance with their endothelial function and that therefore, an incorporation of endothelial function testing could refine platelet function testing. Importantly, these techniques focus on endothelial function in the microcirculation which is especially attractive as as alluded to previously, it appears most likely that the

capillary beds and not the large conduit vessels are the principal site where the many more endothelial cells than platelets exert their influence on platelets. The reasons for this are explained through consideration of the anatomy and physiology of the cardiovascular system. The diameter of a blood platelet is 2-3 μm, the diameter of a capillary is 5-10 μm whereas the diameter of the proximal LAD is around 2.8-4.2 mm. Therefore, the volume to internal surface area ratio of a capillary of 8 μm diameter and 1 cm in length is 0.5 whereas for the proximal LAD of 4 mm diameter and 1 cm length it is 1000. So in capillaries, as compared to large arterial vessels, there is actually a 2000 fold greater ratio of endothelial cells to platelets. In addition, blood flows around 500 times faster in arteries than in capillaries; blood flows in a healthy coronary artery around 10 to over 100 cm/sec but in a capillary at 0.1 cm/sec. Similarly, as outlined in the introduction of this thesis while the cross-sectional area of the aorta is 3-5 cm^2 that of the body's total capillary bed is approximately 4500-6000 cm^2, comprising the vast majority of the total surface area of the circulation [380-382]. Therefore, within capillaries, rather than the large vessels there is the space, time and area for platelets to have intimate interaction with local endothelial cells that cannot be matched in larger vessels and therefore, for the important synergistic interactions described in this thesis to take place. Furthermore, it is suggested that the microvasculature is the initial site of endothelial damage and dysfunction in subjects at risk of cardiovascular disease so the capillaries are of particular importance to my studies. Leading on from the pilot trials of these two techniques it will be interesting next to measure endothelial function in healthy volunteers and patients as an adjuvant to my platelet function studies.

As an additional line of investigation in this thesis I explore the complex interplay of platelets and coagulation in atherothrombosis. This is because both play a critical role in thrombus formation and platelets are also fundamental in regulating the coagulation

system. This is important as configuration of the fibrin network has been shown to be associated with outcome with dense clots predisposed to thrombosis, raising the possibility of clot characterisation and modulation as a therapeutic intervention in atherothrombosis [364]. In order to do this, the effects of standard anti-platelet therapies on platelet activity were determined using traditional tests of platelet function, namely LTA, ATP release, P-selectin and PAC-1 expression. Advanced rheological analyses were then used to establish the influence of these therapies on clot microstructure. Importantly, viscoelastic properties of incipient clots have been shown to be representative of clot architecture and fibrin polymerisation [365]. The novel biomarker, GP was used which defines the point at which fluid blood turns into a viscoelastic solid, incipient clot. From this the fractal dimension (d_f) and relative mass (RM) were calculated as measures of clot complexity and density. Using this new biomarker which has been used to study clot formation in previous studies in different patient populations [383] but never related and validated against platelet reactivity, the effects of platelets and anti-platelet therapies on the microstructure of the fibrin network of incipient clots was determined. This method clearly defined a pre-treatment value of d_f = 1.72 (± 0.05) representative of a normal index of haemostasis, in keeping with previous published studies. Importantly, this correlated with high pre-treatment levels of platelet reactivity, in keeping with previous published cut-off values. Aspirin therapy alone had minimal effects on platelet reactivity reducing only AA and ADP induced platelet aggregation in the LTA but did not affect platelet reactivity through any other testing method, as found in my earlier studies. In keeping with, this aspirin also did not reduce either d_f or RM. In contrast, prasugrel therapy substantially blunted platelet reactivity as assessed by ATP release, P-selectin and PAC-1 expression and all agonists tested in the LTA apart from TRAP-6. Prasugrel therapy also led to significant

reductions in d_f and had a considerable impact on the RM of clots, which was reduced by 40%. These results are in keeping with low dose aspirin being a weak inhibitor of platelet reactivity and prasugrel, which is well known to be a more potent anti-platelet agent. Notably, these studies highlight the intricate link between platelet reactivity and the fibrin architecture of clots with changes in microstructure taking place when platelets were strongly inhibited by prasugrel but not when platelets remained relatively active following aspirin therapy.

DAPT with aspirin plus prasugel or aspirin plus tigacrelor were both associated with strong reductions in aggregation to all agonists tested and also reduced P-selectin expression. Importantly, supporting the link between platelet activity and clot architecture both therapies decreased d_f and led to substantial reductions in the RM of clots of 35% and 45%, respectively. These results are illustrative that stronger inhibition of platelet function is associated with a more porous network of fibrin strands resulting in the formation of less dense clots which, importantly can be detected as changes in d_f. It was also found through projections of RFA's and SEM's that whilst changes in d_f are small they will have a large impact on the overall structure of the fibrin network of clots because of the non-linear relationship between d_f and fibrin mass.

As well as providing further mechanistic insight into the links between platelet reactivity and clot formation, this set of experiments indicate that the therapeutic benefits of anti-platelet therapies may also be linked to their abilities to reduce thrombus density. Results also demonstrate that it is activation of $P2Y_{12}$ receptors that is the key driver of platelet interactions in clot formation, in keeping with the contribution of ADP but not TXA_2 receptors in the initiation of intravascular coagulation [384]. In line with this

conclusion, no significant microstructural change was noted in volunteers taking aspirin 75mg. This is in contrast to a recent study, where 300mg aspirin in patients with ischaemic stroke produced a significant reduction in D_f [385]. Aspirin primarily acts to inhibit platelet COX-1 however, at higher doses it exerts other effects such as acetylation of fibrinogen, prothrombin and other coagulation factors [386, 387]. These direct roles in clot modulation might be seen with higher doses of aspirin (300mg) but perhaps not at lower doses (75mg). It is also notable that addition of aspirin to prasugrel produced no further reduction in markers of clot density despite additional inhibition of platelet TXA_2 pathways. As previously reported, this may be further indicative that in the presence of strong $P2Y_{12}$ receptor blockade aspirin produces little or no additional therapeutic benefit [149, 388].

These results are also in keeping with previous studies suggesting that this novel point of care visco-elastic technique is able to accurately and reproducibly assess clot architecture with the potential of determining the effects of disease and therapeutic intervention on clot quality. Importantly, this technique uses unadulterated whole blood and is measured immediately at the bedside during real-time clot formation, providing a rapid assessment of coagulation that can be more readily performed than current methods [389]. A more thorough understanding of how the fibrin network is structurally organised could be more informative in determining the effects and outcomes of anti-platelet therapies and used in the clinical setting with the potential of individualising therapies.

8.2 Conclusion

To conclude, despite substantial therapeutic developments over the last decade, recurrent thrombotic events and bleeding complications continue to occur, highlighting the need for further optimisation of standard treatment strategies, especially in those patients who remain at increased risk of MACE. The new therapeutic approaches put forward in this thesis share the common goal of optimizing thrombotic risk by reducing not only thrombotic risk but also by reducing bleeding risk. Therapeutic approaches to atherothrombosis are certain to evolve over the next decade and could potentially, take into account some of the fundamental concepts highlighted in this thesis. On the contrary, a wealth of cardiovascular research worldwide continues to attempt to link *ex vivo* platelet function studies in large randomised cardiovascular clinical trials to thrombotic risk and outcomes. Whilst a therapeutic window of platelet reactivity and the tailoring of anti-thrombotic therapy in a personalised therapeutic manner, both based on platelet reactivity are attractive concepts full of apparently obvious potential to improve cardiovascular medicine for patients, are these realistic goals? Randomised trials repeatedly fail to reduce ischaemic complications and improve patient survival through platelet testing and despite intense efforts, no causal relationship between the results of platelet testing and thrombotic events has actually been firmly established. This thesis highlights the exquisitely complicated nature of atherothrombosis exploring not only the key roles of platelets but also the coagulation system and the endothelium. Whilst it is proposed that genotyping as an adjuvant to platelet testing could be helpful, I would further suggest that due consideration needs to be given to both the *in vivo* environment in which platelets reside and a more global representation of thrombosis involving both the cellular and protein parts of the coagulation system is called for. Given

the multifactorial and complex nature of atherothrombosis, whilst therapies will evolve I believe that identification of a single marker predicting thrombotic outcomes beyond traditional and newer risk factors for cardiovascular disease will remain a more challenging goal. A current principal aim of cardiovascular research and medicine today has to focus on the modification and optimisation of risk factors as prevention will always be better than cure.

Chapter 9: References

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